

**UNIVERSIDAD POLITÉCNICA DE MADRID**

Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de  
Biosistemas

Centro de Investigación en Biotecnología y Genómica de Plantas



**Bacterial chemosensory signaling pathways  
and their role in plant pathogenesis**

**DOCTORAL THESIS**

Submitted for the degree of Doctor by:

**Martí Munar Palmer**

BSc. in Biotechnology  
MSc. in Molecular Life Sciences

Madrid, 2024





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**Doctoral Degree in Biotechnology and Genetic Resources of  
Plants and Associated Microorganisms**

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Dr. Emilia Antonia López Solanilla

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*“¡Y ahora me como la tarta,  
claro que me como la tarta,  
me como la tarta y lo que me dé a mí la gana!”*

*Paquita Salas*



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## Abstract

Chemoperception allows bacteria to perceive environmental stimuli and respond accordingly. It is governed by signaling pathways called chemosensory systems, composed of chemoreceptors or MCPs, a histidine kinase CheA, an adaptor protein CheW, and a response regulator CheY. Other proteins come into place for adaptability to stimuli (CheR and CheB) and signal termination (CheZ).

The main objective of this thesis is to uncover the role of chemoperception in the context of bacterial plant pathogenesis using two model plant pathogens: *Pseudomonas syringae* pv. tomato DC3000 (PsPto) and *Dickeya dadantii* 3937 (Dd3937).

In the first chapter, the roles of the multiple chemosensory systems of PsPto were explored in the context of plant pathogenesis. While some bacteria possess a single chemosensory system in charge of governing chemotaxis, others possess more than one system putatively involved in other processes that remain poorly understood. Chemosensory systems are categorized in three functional groups: those that evolve to control flagellar motility (F), type IV pilus-based motility (Tfp), and those involved in alternative (non-motility) cellular functions (ACF). PsPto contains four chemosensory systems, categorized as F6, F8, ACF, and Tfp. Here, the PsPto chemoreceptors were assigned to chemosensory systems and the F and ACF systems of PsPto were deeply characterized. Moreover, the F6 and F8 chemosensory systems of PsPto were visualized for the first time using cryo-electron tomography. Analysis through mutations in the kinase and response regulator for each chemosensory system confirmed how chemotaxis and swimming motility is governed by the F6 system, while the F8 and the ACF systems play a modulating role in swimming motility. Also, the kinase and response regulator from the F6 and F8 chemosensory systems were found to work independently in the regulation of biofilm and twitching motility. Furthermore, it was shown how the F6, F8, and ACF kinases interact with the ACF response regulator WspR, supporting a crosstalk among chemosensory systems. Finally, the implication of these chemosensory systems in regulating bacterial virulence in tomato plants was observed.

In the second chapter, the PsPto-PscC chemoreceptor and its importance in the PsPto pathogenicity was characterized. The importance of chemotaxis in plant pathogenicity has been experimentally proven. However, knowledge on the specific plant components that elicit this response is still scarce. PsPto-PscC was found to bind GABA and L-Pro, two abundant

components of the tomato apoplast. The recognition of both compounds by PsPto-PscC caused chemoattraction to both amino acids and participated in the regulation of GABA catabolism. Mutation of the PsPto-PscC chemoreceptor caused a reduced chemotactic response towards these compounds, which in turn impaired entry and reduced virulence in tomato plants. Since GABA and L-Pro levels significantly increase in tomato plants upon pathogen infection, this exemplifies how bacteria respond to plant signals produced during the interaction as cues to access the plant apoplast and to ensure efficient infection.

In the third chapter, energy taxis was explored in Dd3937 through the analysis of three PAS domain-containing chemoreceptors. Energy taxis is a process by which bacteria perceive their inner metabolic state and control flagellar motility as a response. Dd3937 contains three chemoreceptors structurally homologous to *Escherichia coli* Aer (DDA\_RS17070, DDA\_RS12290, and DDA\_RS12165), which is known to mediate energy taxis in this bacterium. Mutational analysis revealed how DDA\_RS12165 plays a role in supporting optimal growth under rich media and minimal media containing succinate as a sole carbon source. Moreover, DDA\_RS12290 was observed to potentially play a role in sensing oxygen concentration. In terms of pathogenesis, all three chemoreceptors were observed to play a crucial role in regulating symptom development in potato plants.

## Resumen

La quimiopercepción es un proceso que permite a bacterias percibir estímulos ambientales y responder en consecuencia. Está controlado por rutas de señalización llamadas sistemas quimiosensoriales, que se componen de quimiorreceptores, una histidina quinasa CheA, una proteína adaptadora CheW y un regulador de respuesta CheY. También intervienen proteínas adicionales que regulan la adaptación a estímulos (CheR y CheB) y la terminación de la señal (CheZ).

El objetivo principal de esta tesis es estudiar el papel de la quimiopercepción en el contexto de la fitopatogénesis bacteriana utilizando dos patógenos vegetales modelo: *Pseudomonas syringae* pv. tomato DC3000 (PsPto) y *Dickeya dadantii* 3937 (Dd3937).

Primero, se investigaron las funciones de los múltiples sistemas quimiosensoriales de PsPto. Mientras que algunas bacterias poseen un único sistema que controla la quimiotaxis, muchas bacterias poseen más de un sistema, cuyas funciones son aún desconocidas. Estos sistemas se categorizan en tres grupos funcionales: los involucrados en motilidad flagelar (F), en motilidad basada en pili tipo IV (Tfp), y los involucrados en funciones celulares alternativas a la motilidad (ACF). PsPto contiene cuatro sistemas clasificados como F6, F8, ACF y Tfp. Aquí, se asignaron los quimiorreceptores de PsPto a sus diferentes sistemas y se caracterizaron los sistemas de tipo F y ACF. Se visualizaron por primera vez los sistemas F6 y F8 de PsPto mediante criomicroscopía electrónica. El análisis a través de mutaciones en la quinasa y el regulador de respuesta de cada sistema confirmó cómo la quimiotaxis y la motilidad tipo “swimming” están gobernadas por el sistema F6, mientras que los sistemas F8 y ACF desempeñan un papel modulador en la motilidad tipo “swimming”. También se observó cómo la quinasa y el regulador de respuesta de los sistemas F6 y F8 trabajan de manera independiente en la regulación del biofilm y la motilidad tipo “twitching”. Además, se observó cómo las quinasas de F6, F8 y ACF interactúan con el regulador de respuesta del sistema ACF, WspR, lo que confirma una interacción cruzada entre los sistemas quimiosensoriales. Finalmente, se mostró cómo los sistemas F y ACF juegan un papel importante en la regulación de la virulencia en PsPto.

Segundo, se llevó a cabo la caracterización del quimiorreceptor PsPto-PscC y su importancia en patogénesis. La importancia de la quimiotaxis en mediar procesos de entrada a estructuras de la planta está ampliamente demostrada. Sin embargo, los componentes vegetales

específicos que estimulan la entrada todavía no se conocen en profundidad. En este trabajo se determinó que el quimiorreceptor PsPto-PscC une ácido  $\gamma$ -aminobutírico (GABA) y L-prolina (L-Pro), dos componentes abundantes del apoplasto de las plantas de tomate. Se comprobó cómo la quimioatracción de PsPto hacia estos compuestos está mediada por PsPto-PscC, y que dicha interacción afecta a la regulación génica y a la virulencia de PsPto. Estos compuestos aumentan como respuesta defensiva de la planta ante el contacto con un patógeno, por lo que éste es un ejemplo de cómo las bacterias utilizan compuestos vegetales como señal para una infección exitosa.

Por último, se exploró el proceso de taxis energética en Dd3937 a través del análisis de mutantes en tres quimiorreceptores con dominio PAS: DDA\_RS17070, DDA\_RS12290 y DDA\_RS12165. Éstos son estructuralmente homólogos al *Aer* de *Escherichia coli*, conocido por mediar la taxis energética en esta bacteria. Este proceso permite a las bacterias percibir su estado metabólico y modular la motilidad flagelar como respuesta. En este trabajo se observó que DDA\_RS12165 es necesario para el crecimiento óptimo de Dd3937 en diferentes medios de cultivo. También se determinó el potencial rol de DDA\_RS12290 en la percepción de oxígeno. Finalmente, se observó cómo los tres quimiorreceptores están involucrados en la patogenicidad, con gran significancia en el desarrollo de síntomas en plantas de patata.

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## Abbreviations and Acronyms

|          |                                                            |
|----------|------------------------------------------------------------|
| Å        | Angstrom                                                   |
| °C       | Celsius degrees                                            |
| μE       | Microeinstein                                              |
| μg       | Microgram                                                  |
| μl       | Microliter                                                 |
| μM       | Micromolar                                                 |
| 4HB      | Four-helix bundle                                          |
| ACF      | Alternative Cellular Function                              |
| Ap       | Ampicillin                                                 |
| Asp      | Aspartic acid                                              |
| ATP      | Adenosine triphosphate                                     |
| BACTH    | Bacterial Adenylate Cyclase Two Hybrid                     |
| Bp       | Base pair                                                  |
| c-AMP    | 3'-5'-cyclic adenosine monophosphate                       |
| c-di-GMP | Bis-(3' -5')-cyclic dimeric guanosine monophosphate        |
| Cache    | Calcium channels and chemotaxis receptors                  |
| CAT      | Catalytic module                                           |
| Cb       | Carbenicillin                                              |
| CCW      | Counterclockwise                                           |
| CFA      | Coronafacic acid                                           |
| Cfu      | Colony Forming Units                                       |
| CHASE    | Cyclase/Histidine Kinases Associated Sensory Extracellular |
| cm       | Centimeter                                                 |
| Cm       | Chloramphenicol                                            |
| CMA      | Coronamic acid                                             |
| CR       | Chemoreceptor                                              |

|        |                                                                                      |
|--------|--------------------------------------------------------------------------------------|
| CV     | Crystal violet                                                                       |
| CW     | Clockwise                                                                            |
| CWDEs  | Cell wall degrading enzymes                                                          |
| Dd3937 | <i>Dickeya dadantii</i> 3937                                                         |
| Dhp    | Dimerization and histidine phosphotransfer domain                                    |
| DMSO   | Dimethylsulfoxide                                                                    |
| DNA    | Deoxyribonucleic acid                                                                |
| Dpi    | Days post-inoculation                                                                |
| Ec     | <i>Escherichia coli</i>                                                              |
| ET     | Electron tomography                                                                  |
| ETI    | Effector triggered immunity                                                          |
| eV     | Electronvolt                                                                         |
| F      | Flagellar                                                                            |
| FAD    | Flavin adenine dinucleotide                                                          |
| FB     | Flexible bundle                                                                      |
| g      | Grams                                                                                |
| GABA   | $\gamma$ -Aminobutyric acid                                                          |
| GAF    | cGMP-specific phosphodiesterases, adenylyl cyclases, and FhlA                        |
| GHL    | Gyrase B, Hsp90, MutL                                                                |
| Gm     | Gentamycin                                                                           |
| h      | Hours                                                                                |
| H      | Heptads                                                                              |
| HAMP   | Histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein, phosphatase |
| HBM    | Helical bimodular                                                                    |
| His    | Histidine                                                                            |
| HK     | Histidine kinase                                                                     |
| Hpi    | Hours post-inoculation                                                               |
| HPt    | Histidine phosphotransfer                                                            |

|                |                                               |
|----------------|-----------------------------------------------|
| HR             | Hypersensitive response                       |
| HTH            | Helix-turn-helix                              |
| IAA            | Indol-3-acetic acid                           |
| IPTG           | Isopropyl $\beta$ - d-1-thiogalactopyranoside |
| JA             | Jasmonic acid                                 |
| Kb             | Kilobase                                      |
| KB             | King's B                                      |
| Km             | Kanamycin                                     |
| l              | Liter                                         |
| LB             | Lysogeny broth                                |
| LBD            | Ligand binding domain                         |
| LOV            | Light-oxygen-voltage                          |
| LSD            | Least significant difference                  |
| MCP            | Methyl-accepting chemotaxis protein           |
| m <sup>2</sup> | Square meter                                  |
| mg             | Milligrams                                    |
| MH             | Methylation helices                           |
| min            | Minutes                                       |
| mM             | Millimolar                                    |
| NAD            | Nicotinamide Adenine Dinucleotide             |
| nm             | Nanometers                                    |
| OCS            | One-component system                          |
| OD             | Optical density                               |
| PAMP           | Pathogen-associated molecular pattern         |
| PAS            | Pern-Ant-Sin                                  |
| PBP            | Periplasmic binding protein                   |
| PCR            | Polymerase Chain Reaction                     |
| PHY            | Phytochrome-like                              |

|         |                                               |
|---------|-----------------------------------------------|
| Pi      | Inorganic phosphate                           |
| PMF     | Proton motif force                            |
| PRR     | Pattern recognition receptors                 |
| Psa     | <i>Pseudomonas syringae</i> pv. actinidiae    |
| PsPto   | <i>Pseudomonas syringae</i> pv. tomato DC3000 |
| PTI     | PAMP-triggered immunity                       |
| Rif     | Rifampicin                                    |
| RH      | Relative humidity                             |
| RNA     | Ribonucleic acid                              |
| ROS     | Reactive oxygen species                       |
| RR      | Response regulator                            |
| RT      | Room temperature                              |
| RT-qPCR | Reverse transcription quantitative PCR        |
| s       | Seconds                                       |
| SA      | Salicylic acid                                |
| SAR     | Systemic acquired resistance                  |
| SBP     | Solute binding protein                        |
| SD      | Signaling domain                              |
| SEN     | Sensor module                                 |
| SSD     | Signaling subdomain                           |
| Sp      | Spectinomycin                                 |
| T3Es    | Type-III Effectors                            |
| T3SS    | Type-III Secretion System                     |
| Tat     | Twin-arginine transporter                     |
| Tc      | Tetracycline                                  |
| TCA     | Tricarboxylic Acid                            |
| TCS     | Two-Component System                          |
| Tfp     | Type-IV pili                                  |

|       |                                                          |
|-------|----------------------------------------------------------|
| TM    | Transmembrane                                            |
| TRA   | Transduction module                                      |
| vol   | volume                                                   |
| WT    | Wild type                                                |
| wt    | weight                                                   |
| X-gal | 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside |





## **General introduction**



Bacteria and archaea are unicellular microorganisms widely distributed across the planet. Most bacteria are found in the so called ‘big five’ habitats: deep oceanic subsurface, upper oceanic sediments, deep continental subsurface, soil, and oceans. Bacteria also inhabit other environments like groundwater, the atmosphere, the ocean surface microlayer, humans, animals, and the phyllosphere, and despite being fewer by orders of magnitude (Flemming & Wuertz, 2019), their presence is fundamental in nature. For bacteria to be able to colonize every single habitat on Earth, they need to perceive and respond to both internal and environmental cues, adapting constantly to changing conditions. Responses to these can include changes in metabolism, induction of defense structures, production of toxins, virulence, stimulation of motility or induction of a cooperative lifestyle called biofilm (Galperin, 2018).

Bacteria respond to their environment through signal transduction systems. Among the inputs triggering these systems we find temperature, pH, light, oxygen, simple molecules like sugars or amino acids, intracellular redox balance, and even cell shape and integrity (Galperin, 2018). These signals are perceived, and a response is elaborated mostly by three categories of signaling pathways: one-component systems, two-component systems, and one special two-component system that deserves a category itself, the so-called chemotaxis system (Wuichet & Zhulin, 2010).

## **1. Signal transduction pathways**

Until not so long ago, two-component systems were thought to be the most common mechanism to respond external and internal stimuli. Two-component systems (TCS) transduce the signal through phosphotransfer between two key elements: a histidine kinase and a response regulator. However, Ulrich et al. (2005) described how there was a simpler system that was more common in regulating cellular processes in prokaryotes than TCS. One-component systems (OCS) follow the same mechanism as two-component systems. However, instead of having the signal transferred between two different proteins, the system is composed of one single protein containing an input domain fused to an output domain (Figure 1).

Signal transduction pathways modulate cellular processes by regulating transcription using DNA-binding helix-turn-helix (HTH) output domains, by altering the levels of second messengers, or by changing motility patterns in response to chemical stimuli, a process called chemotaxis. In this section, OCS, TCS, and chemotaxis systems will be described thoroughly.

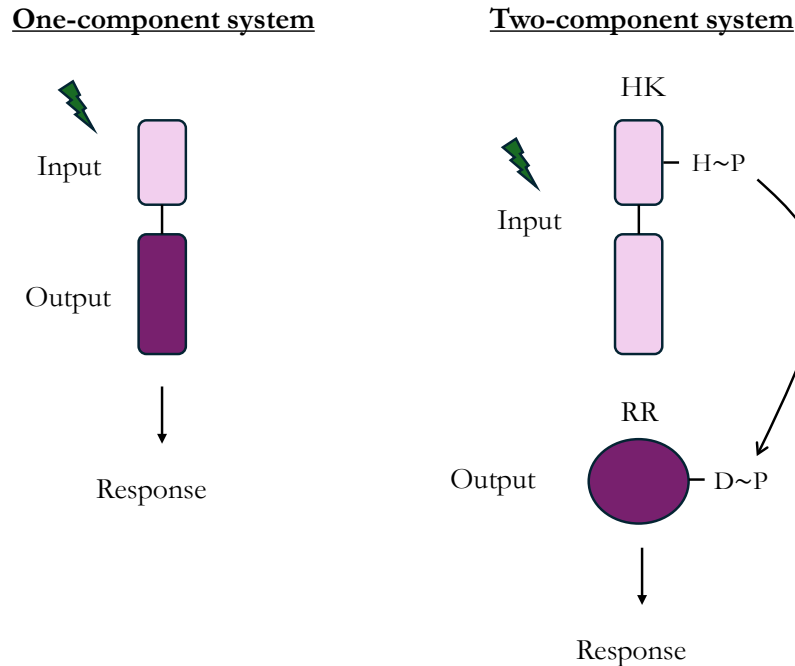


**Figure 1. Schematic view of one-component and two-component systems.** Stimuli are perceived by an input domain (light pink) and a response is given by an output domain (dark purple). HK, histidine kinase; RR, response regulator. Phosphorylation in the histidine (H) and the aspartate (D) residues is indicated by “~P”.

## 1.1. One-component systems

One-component systems (OCS) consist of a single protein containing an input and output domain. Typically, transcription in bacteria is regulated by single proteins containing a ligand-binding domain (input) and a DNA-binding HTH domain (output) (Aravind et al., 2005a). Very well studied examples are the lactose operon repressor (LacI) and the catabolite activator protein (CAP) of *Escherichia coli*. Typical input sensory domains include the Pern-Arnt-Sim (PAS), nitrate- and nitrite-sensing (NIT) domain, *AmiR* and *NasR* transcription antitermination regulators (ANTAR) and calcium channels and chemotaxis receptors (Cache) domains, among others (Galperin et al., 2001; Shu et al., 2003; Shu & Zhulin, 2002; Taylor & Zhulin, 1999). Many of these input domains are also found in TCS. For instance, PAS domains and HTH domains are the input and output domains of the NtrB-NtrC TCS, respectively (Weiss et al., 2002). Contrary to TCS, OCS unite both sensory domains in the same protein without the need for a histidine kinase and a response regulator interaction through phosphorylation. The fact that similar sensory domains are found in both OCS and TCS indicate that both signaling systems can mediate similar signals and responses. However, OCS show greater input and output domain diversity than TCS. In fact, all sensory domains of TCS are present in OCS, but OCS have some exclusive domains. The most common input domains are small-molecule-binding domains, but also enzymatic, cofactor containing, and protein-protein interaction domains (Ulrich et al., 2005).

Interestingly, the number of OCS and TCS increase proportionally with genome size. However, their abundance is also very much dependent on the environment to which bacteria need to adapt. For instance, the genome of the marine bacterium *Trichodesmium erythraeum* and the soil bacterium *Sinorhizobium meliloti* are comparable in size, but they show a great difference in the number of one-component regulators (Ulrich et al., 2005). The latter has nearly 6 times more one-component regulators, presumably because of its complex developmental program (Galibert et al., 2001) and the constant changing microenvironments it must face, such as soil, rhizosphere, and the interior of plant roots. In contrast, *T. erythraeum* has a simpler developmental program and, because it is found in upper levels of tropical oceans, it encounters somewhat steady environmental conditions (Staal et al., 2003).

OCS are thought to be evolutionary precursors of TCS for three primordial reasons: the simplicity of having both input and output domain in the same protein; the wider domain catalogue that OCS present in terms of diversity compared to TCS; and the fact that generally prokaryotes show more OCS than TCS (Ulrich et al., 2005). However, if OCS are simpler and more widely distributed, why is there a need for the emergence of TCS? It is believed that OCS mostly respond to stimuli in the cytosol, as 97% of one-component regulators that have an HTH domain do not have a transmembrane (TM) domain and hence are predicted to be cytosolic proteins. In contrast, histidine kinases from TCS frequently present a TM domain, which indicates that the fundamental difference between OCS and TCS lies on the location of the signal, this being periplasmic or extracellular. Moreover, since most signal transduction systems involve gene regulation, one would speculate that one-component regulators would not be effective in sensing extracellular stimuli and binding DNA. A forward solution to this would be to divide the system in two, enabling the signal to be perceived extracellularly but elaborate a response intracellularly (Ulrich et al., 2005).

## 1.2. Two-component systems

Two-component systems (TCSs) are one of the best studied models of signaling pathways (Gao & Stock, 2009), and they are present in bacteria, fungi and plants. TCSs consist of a sensor histidine kinase (HK) and a cognate response regulator (RR). The HK receives either intracellular or extracellular cues and signals to its cognate RR through a phosphoryl-transfer reaction. This phosphorylation typically causes a conformational change that allows RRs to function, generating the output response (Stock et al., 2000a).

## Histidine Kinases

HKs typically function as homodimeric proteins (Rivera-Cancel et al., 2014). HKs transfer the phosphoryl group from an ATP to a conserved histidine residue. When phosphorylated, the phosphate group is transferred to a conserved aspartate residue of its cognate RR. Finally, RRs will undergo dephosphorylation, this being a critical step in the process to ensure that more signal can be processed. Dephosphorylation can happen due to spontaneous hydrolysis, to specific dedicated phosphatases, or to the phosphatase activity of their cognate histidine kinase when it turns inactive (Gao & Stock, 2017).

HKs are categorized into three classes (I-III) based on their modular design. The first two classes of HKs were described depending on the location of the substrate histidine and their adjacent residues (H-box), in relation to the ATP-binding domain (ABD). Moreover, Class I and Class II HKs function as dimers. Class I HKs comprise most HKs and have both these domains located adjacently (Alex & Simon, 1994). Additionally, Class I HKs are organized in three functional regions structurally differentiated: a sensor module (SEN), a transduction module (TRA), and a catalytic module (CAT). Class II HKs have the H-box and ABD separated in distance. This second class of HKs was created to assign the HK responsible for chemotaxis, CheA, as it is structurally very different from the previously known HKs. In this case, CheA did not appear to have a sensory domain and the H box was located at its P1 domain at N-terminal and separated from the ABD (Bilwes et al., 1999). Class III HKs show shared features between Class I and Class II HKs. Their sensory domain is similar to HKs of Class I, but their organization regarding the H-box and ABD domain resembles that of Class II. In contrast to the other two classes, Class III HKs lack a dimerization domain (Adebali et al., 2017).

Looking closely at the HKs structure, the SEN module is located at N-terminal and harbors one or more sensory domains. These domains are highly diverse, as well as all the signals that HKs can recognize (Stock et al., 2000b). The TRA module consists of two or more helical TM segments (only in TM HKs), and/or one or more signal-transmission domains such as HAMP (histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein, and phosphatase), PAS or GAF (cGMP-specific phosphodiesterases, adenylyl cyclases, and FhlA), among many others (Bhate et al., 2015). The CAT module is the minimal unit for HKs activity, and it is shared among all Class I HKs. It consists of two domains: a dimerization and histidine-phosphotransfer domain (DHp) and an ABD. The DHp is formed by two- $\alpha$ -helix hairpin that drives homodimerization. The substrate histidine is located within a conserved sequence motif (H-Box, mentioned above) approximately in the middle of the DHp (Marina et al., 2005; Tomomori et al., 1999). The ABD consist of a globular  $\alpha/\beta$  domain with a three-helix core packed on one side against a five-stranded  $\beta$  sheet which binds ATP-Mg<sup>2+</sup> (Trajtenberg et al., 2010).

There are variations from the simple modular organization presented above. There are HKs that include a REC domain (found in RRs) in the same protein sequence, named hybrid HKs (HHKs) (Stock et al., 2000a). HHKs usually play a role in signal transduction pathways that involve intermediate proteins containing CAT and receiver (REC) domains found in simple TCS.

## Response regulators

RRs always include a REC domain containing the substrate aspartate residue that is phosphorylated by the cognate HK, causing RR activation. Additionally, RRs can contain other domains, referred as output domains, that define their response, such as DNA-binding, ligand-binding, protein-binding, or enzymatic domains (Galperin, 2010). In fact, most RRs combine the REC domain with one or more output domains. However, the term “response regulator” was first used by Daniel Koshland to describe the CheY chemotaxis protein, which consists only of the REC domain (Koshland, 1977). In bacteria, only 17% of all RRs share this structure. These RRs are known to regulate bacterial motility (CheY), to participate in signaling phosphorelays (Spo0F), and to participate in protein-protein interactions governing cell development and division (DivK and CpdR) (Jenal & Galperin, 2009). One challenge when studying RRs is that sequence identity does not reflect similar functions. For instance, *Bacillus subtilis* Spo0F and *Caulobacter crescentus* DivK share 30% identity. Still, these proteins have completely different roles. A more accurate approach in the study of RRs is based on the identification of their output domains to determine if they bind DNA, RNA, specific ligands, other proteins, enzymes, or even transporters.

Most bacterial RRs contain HTH DNA-binding domains as output domains, with a role in transcription regulation (Aravind et al., 2005b), like the OmpR/PhoB and NarL/FixJ families (Baikalov et al., 1996; Buckler et al., 2002). However, there are other DNA-binding RRs that lack an HTH domain, substituting it for a  $\beta$ -stranded domain (Sidote et al., 2008). This is the case of LytTR domains, which are widely spread in bacteria and are involved in virulence of several pathogens (Galperin, 2008; Nikolskaya & Galperin, 2002).

Apart from RRs that work directly as transcriptional regulators, there is a significant group of RRs containing domains that have enzymatic activities and participate in signal transduction: methyltransferases, adenylate cyclases, diguanylate cyclases, c-di-GMP specific phosphodiesterases, histidine kinases, serine/threonine kinases, and protein phosphatases. Examples of some of these RRs can be the CheB chemotaxis protein or the WspR diguanylate cyclase. CheB is a chemotaxis protein that combines the REC domain with a methyltransferase domain located at C-terminal (Djordjevic et al., 1998; West et al., 1995). It is involved in the adaptation process of chemoreceptors to increasing concentrations of ligands (Martin et al., 2001). WspR is a RR with a diguanylate cyclase domain (GGDEF) whose activation is through phosphorylation and formation of a WspR tetramer. Another

example is the Rrp1, a RR containing a GGDEF domain which has been shown to function as global regulator of cell metabolism in *Borrelia burgdorferi* (Rogers et al., 2009).

There are also RRs with protein-binding domains. One surprising example is the PhyR RRs of *Methylobacterium extorquens*, involved in plant colonization and resistance to heat shock and desiccation (Gourion et al., 2006, 2008). This RR contains a REC domain combined with an N-terminal domain that is very similar to the  $\sigma^E$  (RpoE,  $\sigma^{24}$ ) subunit of RNA polymerase. Despite similarities with a sigma factor domain, this protein was never seen involved in DNA-binding or interaction with RNA-polymerase. The  $\sigma^E$  domain of PhyR was shown to bind an anti-sigma factor, a small protein called NepR, when activated through phosphorylation (Francez-Charlot et al., 2009). Therefore, interaction of PhyR with NepR released a sequestered genuine sigma factor  $\sigma^{EcfG}$ , which was now free to interact with the RNA polymerase to stimulate transcription of stress-related genes.

With time, more and more RRs are being characterized and many appear to have output domains that, despite being well characterized, had not yet been associated with previously reported RRs. Examples are RRs from *Desulfatibacillum alkenivorans* and *Desulfococcus oleovorans* whose output domains are membrane transporters for nitrate, sulfate, and dicarboxylates. Moreover, other described RRs contain metabolic enzymes, ATPases, threonine synthases, nucleoside phosphorylases or sugar transferases as output domains, among others (Galperin, 2010).

### 1.3. Crosstalk among signaling pathways

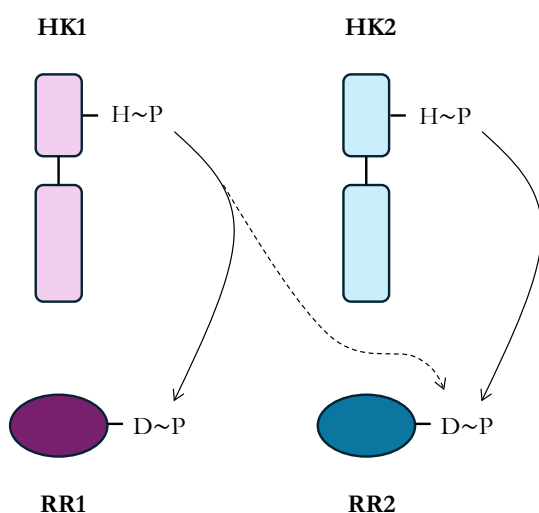


**Figure 2. Crosstalk in signaling pathways.** Dashed line indicates promiscuity between the histidine kinase 1 and the response regulator 2. HK, histidine kinase; RR, response regulator.

Crosstalk among signaling transduction pathways refers to the molecular interaction between two signaling pathways. The term “crosstalk” has been used in the past to describe interactions between pathways that diminish signaling specificity and tend to be avoided by bacteria. It is true that most crosstalk evidence only has been observed after altering bacterial genetics, which lead to the impression that crosstalk might not occur in wild-type organisms. In contrast, many examples of crosstalk have been described as positive scenarios where the interaction between pathways results beneficial for bacteria. This type of beneficial interaction was referred to as “cross-regulation”, in contrast with the previous definition of crosstalk. However, nowadays, any interaction between

signaling pathways is referred to as crosstalk. Therefore, this term will be used throughout the text to refer to any interaction between signaling pathways. Crosstalk predominantly occurs at the level of phosphotransfer between HKs and RRs (Figure 2). However, the source of phosphoryl groups are not always HKs (Laub & Goulian, 2007).

### **Crosstalk as a result of altering bacterial genetics**

When altering bacterial genetics, crosstalk frequently occurs because histidine kinases show promiscuity and are able to phosphorylate many different response regulators. One example is the crosstalk between the PhoR-PhoB and the VanS-VanR TCSs. Studies of these TCSs in *E. coli* revealed that crosstalk only occurred when one of the HKs is absent. More specifically, phosphorylation of PhoB by VanS can only occur when PhoR is absent (Fisher et al., 1995), and PhoR can only phosphorylate VanR when VanS is absent (Haldimann et al., 1997). In each case, crosstalk is a consequence of eliminating the cognate HK. In the case of bifunctional HKs, which have both kinase and phosphatase activities, if crosstalk phosphorylation occurred, the phosphatase activity of the cognate HK would rapidly get rid of the phosphate that was transferred from the non-cognate HK. Therefore, when the cognate HK is removed, crosstalk is revealed. A mathematical model showed that bifunctional HKs are more effective at suppressing crosstalk than monofunctional HKs (only kinase activity). In the latter case, other phosphatases come into place to prevent unwanted crosstalk (Stock et al., 2000a). Apart from phosphatases, crosstalk is also prevented because RRs have higher affinity for their cognate HKs than other RRs, therefore outcompeting noncognate RRs (Laub & Goulian, 2007).

Crosstalk has also been reported to happen when a HK is overexpressed. A remarkable example is that of the overexpression of the NtrB HK in an *E. coli cheA* deficient mutant strain, in which the chemotactic-null phenotype of the mutant strain is suppressed because of NtrB cross-phosphorylating its response regulator CheY, in charge of regulating flagellar rotation. CheY phosphorylation efficiency by NtrB was assessed *in vitro* and it was shown to be significantly lower than by CheA (Ninfa et al., 1988). These examples illustrate a type of crosstalk that is thought not to happen in wild-type cells, but as a consequence of altering bacterial genetics.

### **Crosstalk by acetyl-phosphate**

RRs can also be phosphorylated by different entities than HKs. There is sufficient evidence that acetyl-phosphate, a small phosphor-donor molecule, can also be the source of RRs cross-phosphorylation (Wolfe, 2005). The first finding in this matter was from a study that used *E. coli* cells that expressed the chemotaxis RR CheY but not its cognate HK CheA. Different metabolic scenarios were tested in this genetic background using metabolic inhibitors and generating mutations in acetate metabolism. This study concluded that acetyl-phosphate could activate CheY by phosphorylation and

cover for CheA absence (Wolfe et al., 1988). However, phosphorylation of CheY by acetyl-phosphate is not necessary for chemotaxis itself (Dailey & Berg, 1993), probably because phosphotransfer from CheA to CheY is several times more efficient than from acetyl-phosphate (Da Re et al., 1999; Mayover et al., 1999; Silversmith et al., 1997).

Many researchers have proposed a role for acetyl-phosphate as a global regulator of bacterial behavior in relation to the metabolic state of the cell (McCleary et al., 1993; Wanner, 1992). Supporting this hypothesis, one genetic study linked flagellar and capsule biosynthesis to acetyl-phosphate levels in *E. coli*. In this work, mutations in genes related to acetate metabolism caused high acetate-phosphate levels, which lead to enhanced swimming motility, due to increased flagella biosynthesis. This was confirmed to be caused by cross-phosphorylation from acetate-phosphate to the Rcs phosphorelay (RcsC, RcsB), which activates genes required for capsular biosynthesis (Fredericks et al., 2006).

### **Crosstalk among signaling pathways**

Crosstalk also occurs in wild-type cells resulting in a beneficial trait for bacterial behavior. There is sufficient evidence of crosstalk at the level of RR phosphotransfer. One example is the interaction between the TCSs PhoR-PhoP and YycG-YycF in *B. subtilis* (Howell et al., 2003). Under phosphate limitation, expression of the gene *yocH* is stimulated by PhoR (HK) phosphorylation, but not through PhoP, which is the cognate RR of PhoR. In fact, *yocH* expression is regulated by phosphorylated YycF, whose phosphoryl group actually comes from PhoR. With additional results, a model of crosstalk between these two pathways has been established as a responding mechanism to low phosphate levels (Howell et al., 2006).

Crosstalk has also been suggested to be involved in nitrogen metabolism in *Rhodobacter capsulatus* (Drepper et al., 2006). The NtrB-NtrC TCS regulates nitrogen assimilation and fixation through transcriptional regulation. Cells lacking NtrC are unable to grow with N<sub>2</sub> or urea as a nitrogen source, but cells lacking NtrB are able to grow on these conditions. However, cells lacking both NtrB and an additional HK (NtrY) are unable to grow on N<sub>2</sub> or urea, indicating that NtrY is also phosphorylating NtrC. NtrY takes part in a different TCS together with NtrX as RR. Therefore, this work proves the existence of crosstalk between two different TCS that happens *in vivo* (Drepper et al., 2006).

## **2. Chemotaxis**

Chemotaxis is the directed movement of bacteria towards or opposite gradients of concentration of chemicals. These chemicals can be attractants if they result beneficial for the bacteria, or repellents if they are toxic. As mentioned earlier, this process is controlled by a complex two-component system

signaling pathway, best studied in the enteric bacteria *E. coli* and *Salmonella enterica* serovar Typhimurium (Armitage, 1999; Bren & Eisenbach, 2000).

*E. coli* swim by rotating their flagella in counterclockwise (CCW) direction, which assemble into a bundle that propels the cell forward (run). When the direction of rotation of the flagellar motors is changed to follow a clockwise direction (CW), flagellar filaments disassemble and causes the cell to tumble (Turner et al., 2000) (Figure 3). Bacteria combine these two types of movement, run and tumble, to navigate through the environment depending on the chemicals they encounter. Even though flagella are different among bacterial genus, their swimming behavior relies on changing direction of the flagellar motor.

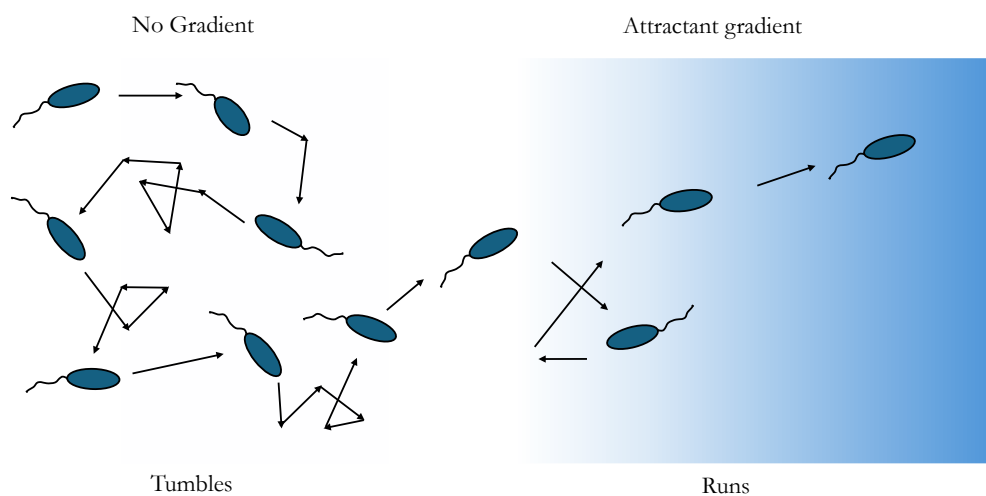


**Figure 3. Schematic view of chemotaxis exhibited by bacteria.** In the absence of attractant (left), bacteria move randomly doing tumbles. In the presence of an attractant gradient (right), bacteria do runs to move towards the source of attraction. Adapted from Bi & Sourjik (2018).

Chemotactic signals are detected by specialized proteins called Methyl-Accepting Chemotaxis Proteins (MCPs), also commonly called chemoreceptors. These proteins are usually transmembrane and localize at cell poles, but they can also be cytoplasmic. These proteins form complexes with the cytoplasmic histidine kinase CheA thanks to the adaptor protein CheW. CheA will transfer the signal to two response regulators that compete for its binding. CheY is the response regulator that, when phosphorylated, will bind to the flagellar motor, more specifically to the FliM switch protein, causing reversals in the direction of the motor rotation (Toker & Macnab, 1997; Welch et al., 1993). CheB is the other response regulator, and it is involved in the adaptation of MCPs to the signal through its methylesterase domain (Anand et al., 1998; Hess et al., 1988). CheR is a protein containing a methyltransferase domain with a constitutive activity, also involved in the adaptation of MCPs. CheZ is a phosphatase that removes phosphate groups from phosphorylated CheY to allow rapid termination of the signal (McEvoy et al., 1999a) (Figure 4).

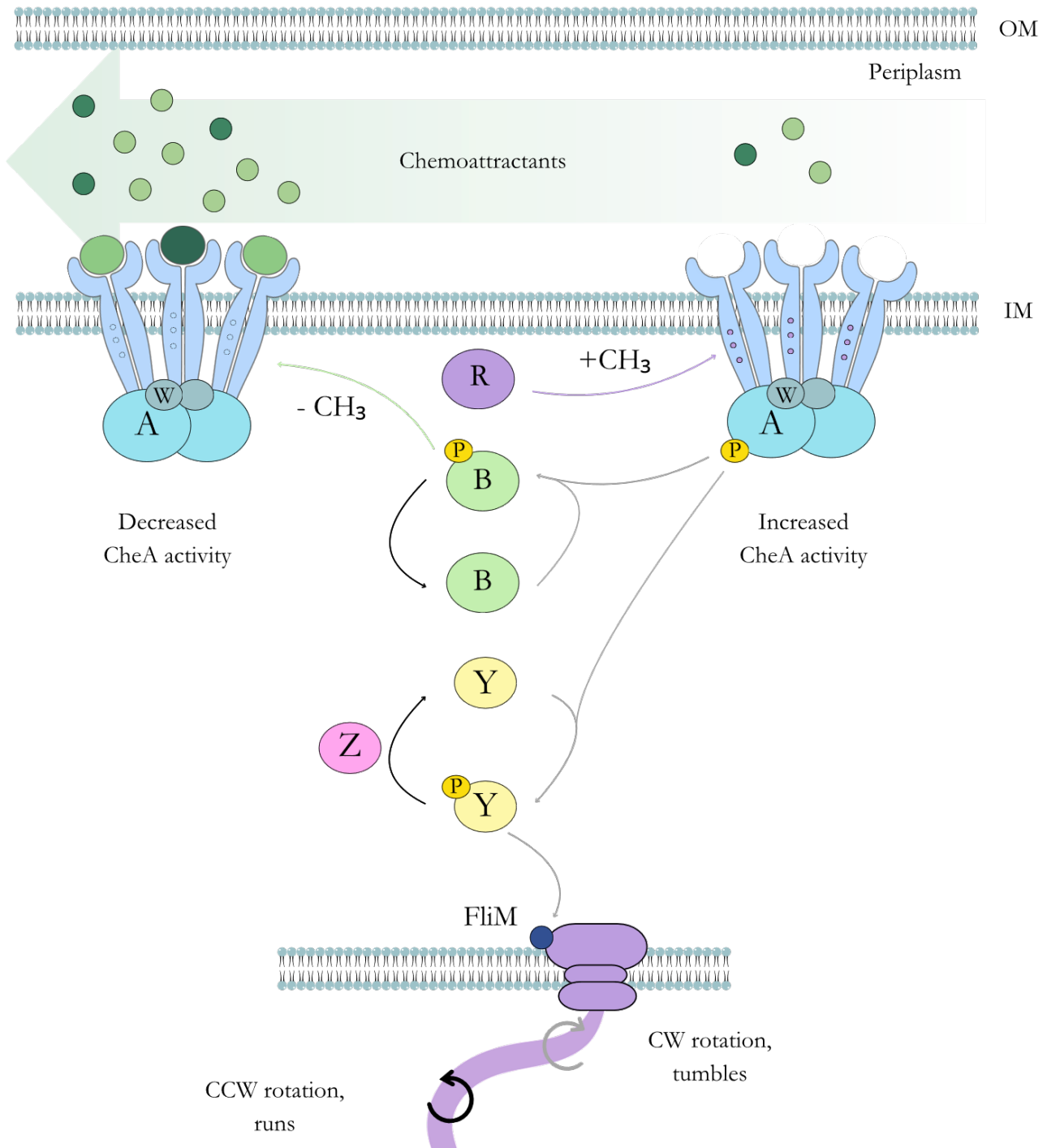


**Figure 4. Chemotaxis signaling pathway.** Changes upon ligand recognition causes a change in the phosphorylation (P) of CheA, causing in turn a change in the phosphorylation ratio of CheY, which in turn modulates flagellar rotation. CheR transfers methyl groups (purple arrow and purple dots) to the MCP signal domain of chemoreceptors, while CheB removes methyl groups (green arrow) upon activation by phosphorylated CheA. Grey arrows represent the signaling cascade in the absence of attractant. Black arrows represent the signaling cascade in the presence of attractant. Che proteins are labelled only with their letter. Only the main Che proteins are included in this scheme. OM, outer membrane; IM, inner membrane; CW, clockwise; CCW, counterclockwise.

In the absence or decrease in attractant concentration, MCPs decrease the amount of signal binding, which stimulates CheA autophosphorylation. As CheA-P phosphorylates CheY, this results in an increase of CheY-P, which binds the flagellar motor causing bacteria to change direction and tumble. CheZ terminates the signal by removing the phosphate from CheY-P. CheA-P also phosphorylates CheB, which stimulates its methylesterase activity, resulting in higher demethylation of MCPs. Demethylated MCPs reduce CheA autophosphorylation rate, even when the concentration of attractant is very low.

The presence or an increased concentration of attractant causes signal binding by MCPs, which undergo a conformational change that inhibits CheA autophosphorylation. This reduces the phosphorylation rate of CheY, causing the rate of direction changing to drop. This causes the bacteria to swim in a specific direction for longer time, what we call “runs”. The ratio of CheB phosphorylation also drops, which decreases demethylation of MCPs. In this scenario, the constitutive methyltransferase CheR continues adding methyl groups to the MCPs, resulting in highly methylated MCPs. This stimulates the autophosphorylation of CheA, even in the presence of attractant, which makes the system go back to pre-stimulus levels.

Chemoreceptors associate with the chemotaxis proteins CheA and CheW to form trimers of receptor dimers, forming hexagonal supramolecular structures called chemotaxis arrays. This structure will be discussed in detail in later sections.

## **2.1. Chemoreceptors or MCPs**

Chemoreceptors are very widespread proteins that can be found in both archaea and bacteria. A typical MCP contains 3 important regions: (1) a transmembrane signaling module, containing the ligand binding domain (LBD) with the ligand binding pocket, and a transmembrane domain; (2) a signal conversion module containing the histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein and phosphatase (HAMP) domain; (3) the kinase control module, containing methylation helices (MH bundle), flexible bundle (FB), and a protein contact region, also called signaling subdomain (SSD) (Alexander & Zhulin, 2007; Hazelbauer et al., 2008; Krell et al., 2011) (Figure 5). MCPs will perceive external stimuli through the LBD and will transmit the signal to the chemotaxis proteins through their cytoplasmic domains (Bi & Lai, 2015; Wadhams & Armitage, 2004). Interestingly, while some MCPs recognize ligands directly through their LBD (Liu et al., 2015; Milburn et al., 1991), others do so aided by periplasmic binding proteins (Kossmann et al., 1988; Machuca et al., 2016). It is important to note that MCPs do not appear isolated, in fact they arrange together with CheA and CheW forming a very complex macromolecular structure, which will be explained in detail later on.

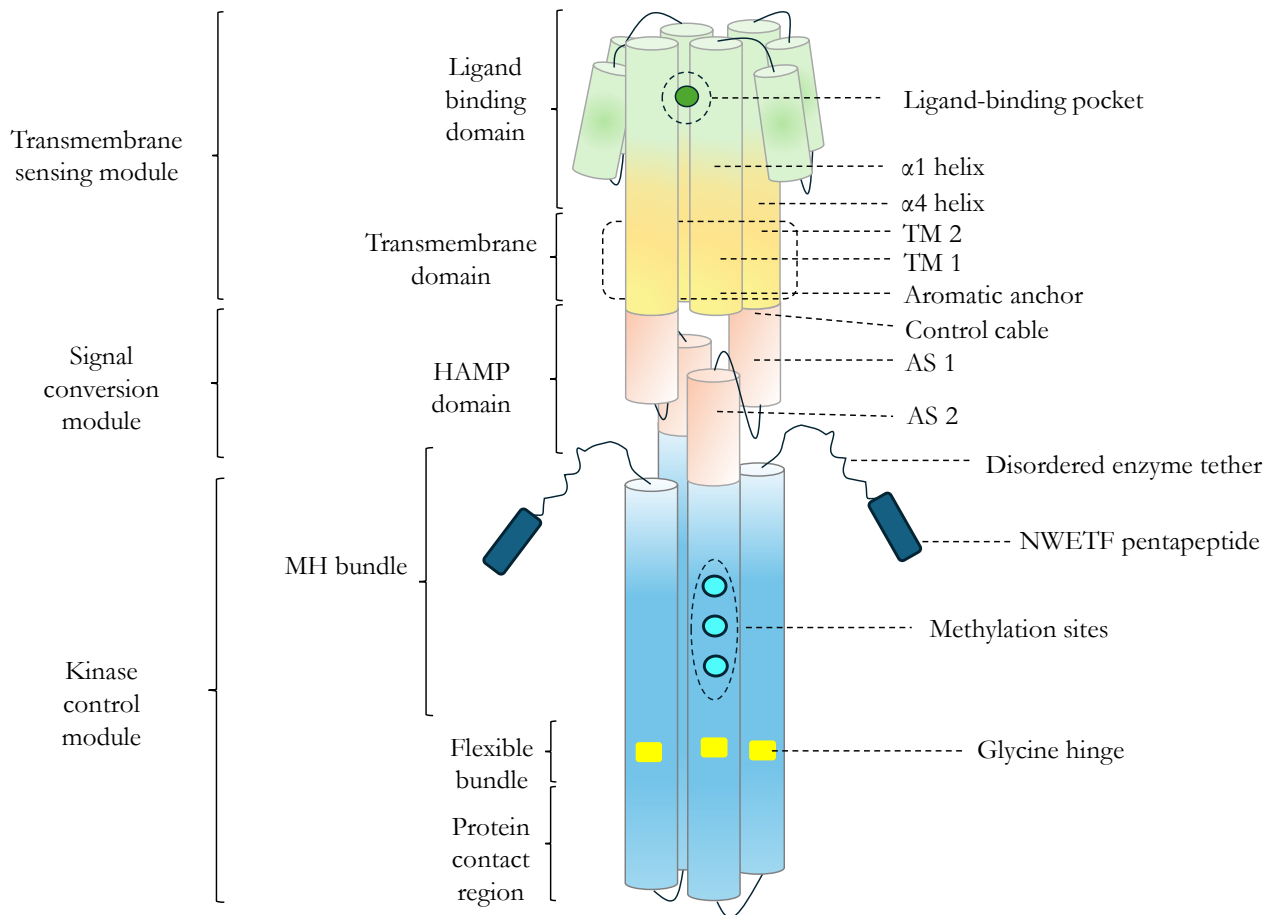


**Figure 5. Structure of a canonical chemoreceptor.** Schematic illustration of the structural features and functional modules of a canonical chemoreceptor homodimer. Adapted from Bi & Lai (2015).

MCPs mediate the recognition of both extracellular and intracellular cues, thus they can be found both embedded in the membrane or in the cytoplasm (Briegel et al., 2014; Lacal et al., 2010). Those containing a TM domain typically sense extracellular molecules and display periplasmic LBDs, whereas entirely cytoplasmic MCPs typically sense the energetic state of the cell. However, some chemoreceptors may contain a cytoplasmic LBD even though they present a TM domain. MCPs were classified into four major classes (I-IV) depending on their membrane topology (Zhulin, 2001). More subclassifications have been done after the previously mentioned, and finally seven categories of MCPs have been established (Ia, Ib, II, IIIIm, IIIc, IVa, and IVb) taking several things into account, like the presence of absence of an LBD domain or the number of TM helices (Lacal et al., 2010; Wuichet et al., 2007) (Figure 6).

A canonical class I MCP contains a periplasmic LBD, a TM domain, and a cytosolic signaling domain (SD) or kinase control module; and they are a predominant class of chemoreceptors in bacteria (Lacal et al., 2010). Depending on the number of TM helices, they are categorized into subclasses Ia, with

two TM helices; and Ib, with one TM helix. The subclass Ia is more common than the Ib, and their main difference is that class Ia MCPs contain an N-terminal TM domain that the Ib MCPs lack.

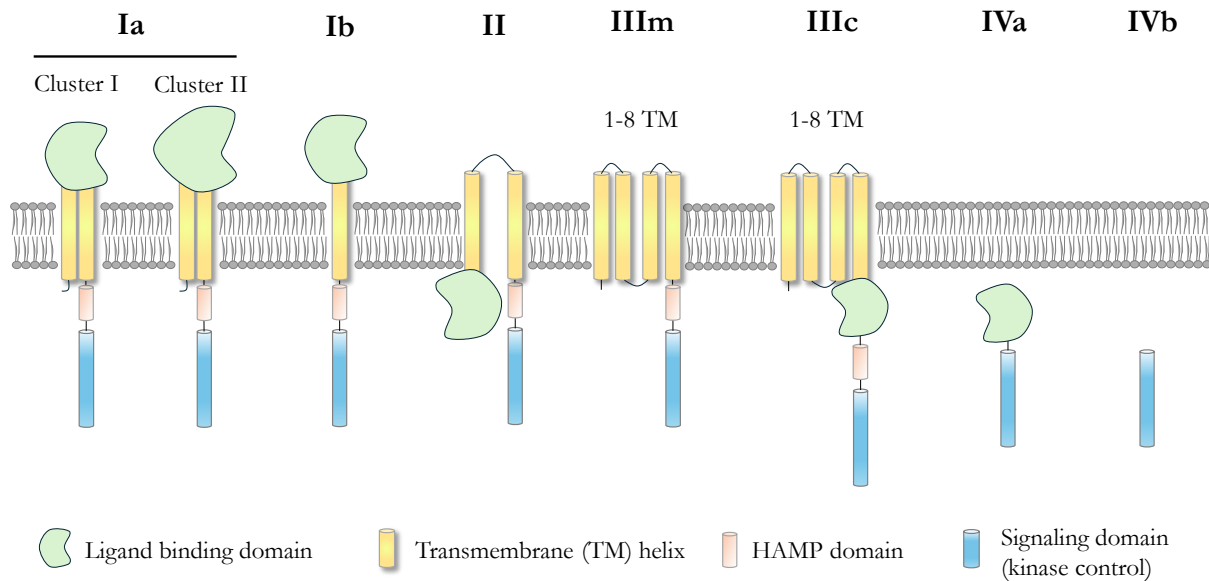


**Figure 6. Classification of methyl-accepting chemotaxis proteins based on membrane topology and LBD.** The larger size of the LBD represents a longer amino acid sequence. Adapted from Salah Ud-Din & Roujeinikova (2017).

Class Ia chemoreceptors have been further subcategorized into two clusters depending on their LBD size. Cluster I Class Ia MCPs harbor an LBD of approximately 120-215 amino acids (aa) and are the most common. In contrast, Cluster II Ia MCPs harbor larger LBD of approximately 215-299 aa (Lacal et al., 2010).

Class II MCPs contain an N-terminal cytoplasmic LBD followed by two TM helices, a cytoplasmic HAMP region and a cytoplasmic SD. This type of MCPs are only found in bacteria and are considerably less common than the rest of MCPs. They are known to mediate aerotaxis (taxis to  $O_2$ ) or energy taxis. One example of class II MCP is the Aer chemoreceptor of *E. coli* (Watts et al., 2004), which contains an N-terminal cytoplasmic PAS domain (Amin et al., 2006).

Class III MCPs contain a number of TM helices that can range from 1 to 8. Their sensor element is located within the membrane part (IIIIm) or in the cytoplasm after the last TM helix (IIIC), followed by the cytoplasmic HAMP region and SD (Lacal et al., 2010; Wuichet et al., 2007). They are more commonly found in archaea than in bacteria. Examples of a bacterial class III MCPs are the KinB (IIIIm) and KinD (IIIC) chemoreceptors of *B. subtilis*, involved in sporulation and bacterial survivor in harsh environments, respectively (Fabret et al., 1999; Wu et al., 2013).

Class IV MCPs group cytoplasmic MCPs that may or may not contain an identifiable LBD (IVa and IVb subclasses, respectively) (Lacal et al., 2010). As well as class III MCPs, they are more frequent in archaea than in bacteria. One example of a bacterial class IVa MCP is the HemAT aerotaxis chemoreceptor from *B. subtilis* (Zhang & Phillips, 2003), which contains a globin motif in its LBD. Class IV chemoreceptors are also found in *Rhodobacter sphaeroides* (Wadhams et al., 2002) and *Vibrio cholerae* (Briegel et al., 2014).

### **Structure of canonical MCP**

As mentioned earlier, a canonical MCP typically contains a periplasmic-sensing or ligand-binding domain (LBD), followed by a transmembrane region, the signal conversion module (HAMP domain), and a kinase control module or signaling domain.

#### Ligand-binding domain

Signals bind to chemoreceptors through the LBD, either directly or in complex with a periplasmic ligand binding protein. While the cytosolic fragments of chemoreceptors are highly conserved, the sequence diversity in LBDs is enormous. A study from 2017 identified 80 different types of LBD (Ortega, Zhulin, & Krell, 2017), and novel chemoreceptors with different LBDs continue being uncovered (Elgamoudi et al., 2021). In many cases, a given LBD type is present in different families of signaling receptors (Amikam & Galperin, 2006; Nikolskaya & Galperin, 2002; Shu et al., 2003; Upadhyay et al., 2016), which supports the hypothesis that LBDs are individual modules that can be rearranged with different signaling modules to generate novel proteins with distinct functions (Matilla et al., 2022). It could be hypothesized that LBD diversity is necessary for recognizing all the different signal molecules that exist. However, LBDs with different structure have been shown to bind the same molecules. A central question in the field of signal transduction is to understand the correlation, if there is any, between the LBD types and the signal molecules they recognize (Matilla et al., 2022).

As mentioned above, LBDs can be classified into cluster I and cluster II depending on their size. Subsequent structural studies suggested that cluster I LBDs contain a single structural module, whereas cluster II LBDs have a bimodular arrangement (Liu et al., 2015; Nishiyama et al., 2016; Pineda-Molina et al., 2012). Different types of LBDs from cluster I and cluster II are presented in Figure 7.

Cluster I LBDs adopt very diverse tridimensional topologies including four-helix bundle (4HB), Per-Arnt-Sim (PAS), cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA (GAF), single calcium channels and chemotaxis receptors (sCache), and cyclases/histidine kinases associated sensory extracellular (CHASE).

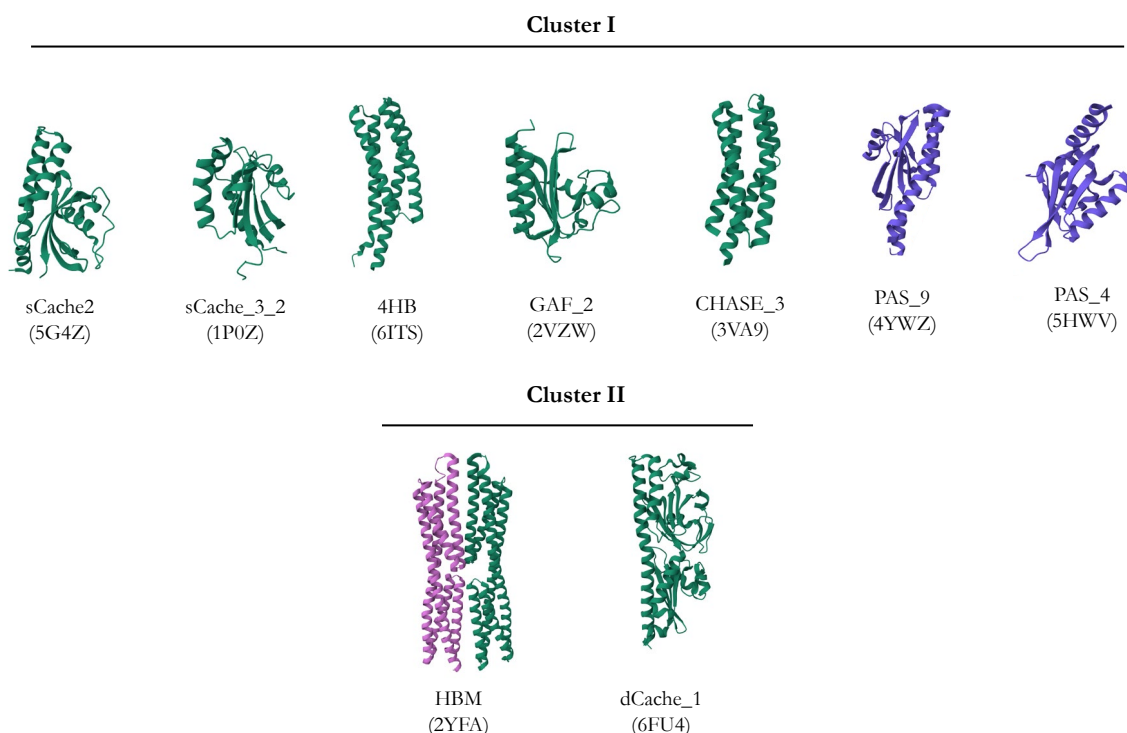


**Figure 7. Diversity of chemoreceptor ligand-binding domains.** Domains in green alone or green and pink are extracellular, whereas domains in purple are cytosolic. LBDs with two chain colors indicate that the domain was experimentally shown to be dimeric. The Protein Data Bank ID for each LBD is indicated in parenthesis.

Two of the most well-known chemoreceptors are those from *E. coli* that bind aspartate and serine (Tar and Tsr), which present a 4HB LBD type (Kim et al., 1999; Krikos et al., 1983; Mise, 2016). However, this LBD type has been shown to bind signal molecules other than amino acids. For instance, the Tcp chemoreceptor from *S. enterica* sv. Typhimurium binds organic acids like citrate and citrate/metal ion complexes (Iwama et al., 2006). Some even bind inorganic phosphate (Pi) directly, like CtpH from *Pseudomonas aeruginosa* (Rico-Jiménez et al., 2016). Other chemoreceptors with 4HB domains have been shown to bind cyclic carboxylic acids (Luu et al., 2015), organic acids and aromatic compounds (Huang et al., 2016), and TCA cycle intermediates (Ni et al., 2013).

PAS domains are very widely distributed among proteins involved in signal perception. They are present exclusively in the cytoplasmic compartment and thus identify signal molecules that come from the inside of the cell. Ligands have been identified for at least six PAS domain families, including PAS, PAS\_4, PAS\_6, PAS\_8, PAS\_9, and PAS\_10; although this classification is currently being revised. Some of these domains contain cofactors like heme for gas sensing (García et al., 2017), FAD and NAD<sup>+</sup> for redox sensing (Ukaegbu & Rosenzweig, 2009; Zhou et al., 2018) or FMN for light sensing (Nash et al., 2011). Other PAS domains have been shown to bind signal molecules directly, such as aromatic hydrocarbons (Busch et al., 2007), Zn (II) (Monk et al., 2019), dodecanoic acids (An

et al., 2014), aromatic amino acids (Wilson et al., 1995), or compounds with autoinducer function (Huang et al., 2020).

GAF types of LBDs are present mostly in sensor histidine kinases, like the phytochrome-like proteins, where they have been shown to bind biliverdin and phycocyanobilin (Bhoo et al., 2001). The bacterial second messenger c-di-GMP has also been described as a ligand of GAF domains. Finally, gases like O<sub>2</sub>, CO, or NO were also found to be recognized by this type of LBDs (Basudhar et al., 2015; Cho et al., 2009; Lee et al., 2008; Sardiwal et al., 2005; Sousa et al., 2007).

sCache domains were often mistaken for PAS domains in the literature. However, as mentioned before, PAS domains have been demonstrated to be exclusively cytosolic. Therefore, all extracytosolic domains annotated as PAS are in fact sCache domains (Upadhyay et al., 2016). One example of this type of chemoreceptor is the TlpB from *Helicobacter pylori*, which binds urea (Sweeney et al., 2012), allowing the bacteria to enter the epithelial tissue (Huang et al., 2015). Another example is the McpP of *Pseudomonas putida* KT2440, which binds the C2 and C3 carboxylic acids acetate, pyruvate, propionate, and L-lactate (García et al., 2015).

So far, the only chemoreceptor containing a CHASE type of LBD has been described to respond to pyrene in *Novosphingobium pentaromativorans* US6-1 (Li et al., 2021).

Cluster II LBDs include the helical bimodular (HBM) and double Cache domains (dCache) (Liu et al., 2015; Milburn et al., 1991; Ortega & Krell, 2014; Pineda-Molina et al., 2012; Upadhyay et al., 2016; Zhang & Hendrickson, 2010).

The HBM domains are composed of two four-helix bundle modules which recognize signals collectively (Pineda-Molina et al., 2012). The physiological relevance of the bimodular arrangement is not completely clear, but it is thought to allow binding of multiple signals, permitting a fine-tuning of the output response (Matilla et al., 2022). One example is the McpS from *P. putida*, which contains a 258-aa LBD that folds into an HBM structure. This chemoreceptor is responsible for chemotaxis towards tricarboxylic acid cycle (TCA) intermediates and butyrate (Pineda-Molina et al., 2012). Amino acid conservation in the ligand binding sites was observed when all sequences of LBDs from the HBM family were aligned, hypothesizing that all HBM LBDs are responsible for organic acids recognition (Ortega & Krell, 2014). Many HBM chemoreceptors have been characterized and support this hypothesis. For instance, McpQ from *P. putida* KT2440 binds citrate or citrate/metal ion complexes (Martín-Mora et al., 2016); and McfS from *P. putida* F1 (Lacal et al., 2010; Pineda-Molina et al., 2012) and McpS from *P. fluorescence* Pf0-1 mediate chemotaxis to organic acids (Oku et al., 2014; Paraless et al., 2013).

The dCache domains are the most abundant extracellular sensor domains (Upadhyay et al., 2016; Zhang & Hendrickson, 2010), and most LBDs experimentally characterized fall into this category (Matilla et al., 2022). While the vast majority of dCache LBDs bind their ligand in their membrane distal module (Cheung & Hendrickson, 2008; Corral-Lugo et al., 2018; Gavira et al., 2018, 2020; Glekas et al., 2012; Nishiyama et al., 2016; Shrestha et al., 2018; Takahashi et al., 2019; Ud-Din et al., 2020; Wu et al., 2013; Zhou et al., 2008), only the TlpC chemoreceptor was found to bind its ligand, lactate, through the membrane proximal module (Machuca et al., 2017). DCache LBDs appear to be the primary amino acid sensors. This might seem contradictory with the fact that the most primary work on amino acid perception came from the characterization of Tsr and Tar chemoreceptors from *E. coli*, which present a 4HB LBD. In fact, among chemoreceptors with 4HB LBDs, only their homologues in *S. enterica* sv. Typhimurium were identified as amino acid-binding chemoreceptors. In contrast, 19 chemoreceptors have been identified so far as amino acid sensors, all containing dCache LBDs. Moreover, these show a wide phylogenetic distribution, suggesting that the amino acid perception trait is more intrinsic of dCache than of 4HB LBDs (Matilla et al., 2022). There are many examples of dCache chemoreceptors. For instance, McpG from *P. putida* KT 2440 is a specific chemoreceptor for  $\gamma$ -aminobutyrate (GABA); McpU binds to putrescine, spermidine and cadaverine; McpA binds to 12 L-amino acids (Corral-Lugo et al., 2016; Reyes-Darias et al., 2015), and McpH was found to respond to five metabolizable purines (Fernández et al., 2016). In addition, chemoreceptor PsPto-PscA from *Pseudomonas syringae* pv. tomato DC3000 has been characterized to bind and mediate chemotaxis to D-Asp, L-Asp, and L-Glu (Cerna-Vargas et al., 2019).

### HAMP domain

This region was first observed as a “linker” between the periplasmic region and the cytoplasmic region of a chemoreceptor. Peter Ames and John Parkinson, when describing locked signal output mutants of the *E. coli* Tsr, suggested that this “linker” was responsible for transmitting conformational changes from the LBD domain to the cytoplasmic domain (Ames & Parkinson, 1988). This linker earned the name HAMP (Aravind & Ponting, 1999) after homologous elements were observed in histidine kinases (Collins et al., 1992), adenylyl cyclases, other methyl-accepting chemotaxis proteins, and certain phosphatases. Certainly, HAMP domains are found in both transmembrane and cytoplasmic signaling proteins (Dunin-Horkawicz & Lupas, 2010).

HAMP domains in bacterial chemoreceptors are 50-residue regions involved in signal transmission, and are composed of two amphiphilic helices (AS1, AS2) joined by a 14-amino acid long flexible connector (Butler & Falke, 1998; Hulko et al., 2006). These structural elements are conserved along the HAMP domain and likely organize into 4-helix bundles in homodimeric signaling proteins (Airola et al., 2010; Butler & Falke, 1998; Hulko et al., 2006; Swain & Falke, 2007; Watts et al., 2008). The

HAMP domain transmits the signal from the LBD to the SD through conformational changes that originate from rotating all helices in a concerted way (Dunin-Horkawicz & Lupas, 2010; Hulko et al., 2006).

It has been elucidated that HAMP domains can cause locked kinase activation or locked kinase inhibition only by replacing a single amino acid and by cysteine-targeted disulphide bonds (Parkinson, 2010). Moreover, HAMP domains can be exchanged between different MCPs without compromising their cellular function. This suggests a common mechanism for signal transduction from the input region to the output region (Ames et al., 2014). Interestingly, not all chemoreceptors have the same number of HAMP domains, even if they are involved in the same process in different bacteria (Dunin-Horkawicz & Lupas, 2010). For example, the cytosolic aerotaxis receptor from *P. aeruginosa* contains five HAMP domains (Airola et al., 2013), while the chemoreceptor responsible for aerotaxis in *E. coli* contains only one HAMP domain (Ma et al., 2005).

#### Kinase control module

The kinase control module is also called cytoplasmic signaling domain (SD), and it is the most conserved element of MCPs. It forms two helices connected through a hairpin turn at the membrane-distal end. These two helices comprise an adaptation or methylation subdomain, a flexible bundle, and protein-contact region, also called signaling subdomain (SSD). The most conserved subregion of the SD is the SSD; whereas the adaptation subdomain shows a moderate degree of conservation, and the flexible bundle is the least conserved subregion of the SD (Alexander & Zhulin, 2007). MCPs have been categorized according to the number of heptads (H) present in the SD and they have been grouped according to their abundance in the bacterial world into seven major (44H, 40H, 38H, 36H, 34H, 28H, and 24H) (Figure 8) and five minor (48H, 42H, 52H, 58H, and 64H) classes (Alexander & Zhulin, 2007). A heptad can be defined as seven consecutive residues (*a-b-c-d-e-f-g*) symmetrically located on each side of the harpin turns, conforming an  $\alpha$ -helical bundle (Alexander & Zhulin, 2007). The seven major classes chemoreceptors represent the 90% out of the more than 2000 MCPs analyzed.

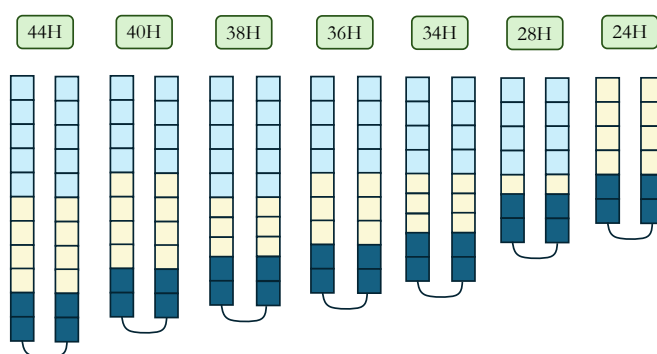


**Figure 8. Schematic classification of chemoreceptors according to the number of heptads in their cytoplasmic signaling domain.** Each colored box represents two heptads. Heptads that belong to different subdomains are represented in colors: methylation subdomain (light blue), flexible bundle (light yellow), and SSD (dark blue). Adapted from Salah Ud-Din & Roujeinikova (2017).

Methylation of chemoreceptors occurs in crucial residues located into the methylation subdomain. This reversible methylation process will lead to signal adaptation of chemoreceptors. Most bacteria contain the consensus sequence -[A/S/T/G]-[A/S/T/G]-X-X-[E/Q]-[E/Q]-X-X-[A/S/T/G]-[A/S/T/G]-, where one of the two consecutive Glx (E/Q) residues is the methylation site (Alexander & Zhulin, 2007; Terwilliger et al., 1986). Methylation and demethylation are carried out by the CheR and CheB chemotaxis proteins, respectively. Chemoreceptors can also be deamidated, which is done by the CheD chemotaxis protein (Callahan & Parkinson, 1985; Kristich & Ordal, 2002; Sherris & Parkinson, 1981).

The flexible bundle subdomain connects the methylation subdomain with the SSD (Alexander & Zhulin, 2007). Despite this subdomain being the least conserved region of chemoreceptors, it still has some conserved features, like the glycine hinge located in the middle of the flexible bundle. This glycine hinge plays a role in the assembly of chemoreceptors into trimers or dimers, which will then form the supramolecular structure together with CheA and CheW (Parkinson et al., 2015). Another conserved feature is the hydrophobic knob residues *a* and *d* of the heptads, which interactions define the conformation of the  $\alpha$ -helical bundle.

The SSD is located at the tip of the cytoplasmic signaling domain and interacts with CheA and CheW. This interaction allows transmission of the signal from the LBD to CheA, which will ultimately modulate flagellar rotation (Parkinson et al., 2015).

## 2.2. The kinase CheA

CheA is the central histidine kinase of the chemotaxis signaling pathway. As mentioned before, CheA is a class II HK, and contains 5 different domains (P1-P5).

The P1 domain is a histidine phosphotransfer (HPT) domain that consists of five helices (A-E) (Quezada et al., 2004; Zhou et al., 1995). HPT domains transfer phosphate groups to histidine residues via phosphorylation. Extensive work has been done in elucidating the structural features of CheA in model organisms like *E. coli* or *Thermatoga maritima*. H48 and H45 are the substrate histidine residues of CheA from *E. coli* and *T. maritima*, respectively. Key aspects of P1 autophosphorylation and phosphotransfer activities have been elucidated, including the association of P1 with CheY (the RR) bound to P2 (Mo et al., 2012). However, the interaction between P1 and P4 has not been fully comprehended yet, despite many efforts both from a computational and experimental approach (Gloor & Falke, 2009; Greenswag et al., 2015; Nishiyama et al., 2014; Zhang et al., 2005).

The P2 domain tethers CheY and CheB to CheA (Li et al., 1995; McEvoy et al., 1999b; Morrison & Parkinson, 1994). It is not essential for phosphotransfer, but it greatly enhances its rate (Stewart et al.,

2000). In fact, mutant strains expressing a CheA lacking the P2 domain still retain chemotaxis, although tumbling responses are impaired (Jahreis et al., 2004). This was found to be overcome by overexpressing the P1 domain. Therefore, the P2 domain is thought to function in increasing the concentration of CheY near the phosphorylated P1 domain (Jahreis et al., 2004).

P1 also interacts with the kinase core, composed of the P3-P4-P5 domains, specifically through the P4 domain. Covalent linking of the P1 domain with the kinase core was found not to be essential for phosphorylation *in vitro* or *in vivo*. However, P1 needs to be overexpressed to maintain chemotaxis fully functional *in vivo* (Garzón & Parkinson, 1996; Nishiyama et al., 2014). Therefore, Muok and collaborators suggest that the linker present in the P1 domain might be important for increasing the local concentration of P1 near the kinase core, a similar situation to the P2 domain's role in tethering CheY and CheB (Muok et al., 2020).

The P4 kinase domain is composed of seven  $\alpha$ -helices and a five-stranded  $\beta$ -sheet that bind  $Mg^{2+}$ -ATP (Bilwes et al., 2001).  $Mg^{2+}$  is required for histidine activation, and significantly increases nucleotide affinity. Crystal structures of the P4 domain with or without nucleotide revealed that the conformation of the ATP-lid changes dramatically upon nucleotide presence. Molecular dynamics studies of linked P1 and P4 complexes suggest that spatial orientation of the ATP molecule induces conformational changes within the ATP-lid that facilitate the interaction with P1 (Zhang et al., 2005).

The P5 domain of CheA and the CheW protein are paralogs that form hexagonal ring structures when they associate with chemoreceptors to arrange into chemotaxis arrays. The P5 domain consists of two intertwined five-stranded  $\beta$ -barrels, designated subdomains 1 and 2, and each subdomain assembles with a CheW in a mirrored manner (Frank et al., 2016; Piñas et al., 2016; Zhao & Parkinson, 2006a, 2006b).

## 2.3. CheY

CheY is a single REC domain structured in five  $\alpha$ -helices and five- $\beta$  sheets. It is phosphorylated by CheA on the conserved Asp57 residue. Upon phosphorylation, CheY-P can interact with FliM, situated in the switch complex of the flagellar motor (Bren & Eisenbach, 1998; Da Re et al., 1999; Sagi et al., 2003; Wemmer et al., 2001). Even though phosphorylation does not significantly change the protein structure, the exposed molecular surface changes, enabling interaction with the flagellar motor (Wadhams & Armitage, 2004). The biological consequence of CheY-P interaction with FliM is the change from CCW rotation to CW rotation, causing bacteria to tumble and change direction (Sagi et al., 2003; Welch et al., 1993).

As mentioned earlier, CheY can also be phosphorylated by acetyl-phosphate. In addition, CheY can also be acetylated by acetyl-CoA synthetase, which has the same effect as phosphorylation (Barak & Eisenbach, 2001). Acetylation levels are high *in vivo* (Yan et al., 2008), and this feature is thought to be involved in adaptation to attractant stimulation (Baron & Eisenbach, 2017), which adds a layer of regulation to the chemotaxis system. However, even though there is some work done in the biochemistry of acetylation in CheY (Barak et al., 2004; Baron & Eisenbach, 2017; Fraiberg et al., 2015; Li et al., 2013; Ramakrishnan et al., 1998; Yan et al., 2008), the regulation aspect of this feature is still very much unknown.

## 2.4. CheB

Together with CheY, CheB is the second response regulator of CheA. Unlike CheY, but in sync with many other response regulators, CheB has a bimodular structure. It has an N-terminal regulatory domain (receiver module) linked to a C-terminal effector domain (output module). CheA activates CheB by phosphorylating an aspartate residue in its N-terminal domain. When active, CheB demethylates chemoreceptors through its C-terminal domain. The catalytic activity of the C-terminal domain was found to remain constitutive in the absence of the N-terminal domain, which illustrates how phosphorylation of the N-terminal domain is responsible for regulating C-terminal activity (Lupas & Stock, 1989). When CheB is not phosphorylated, both modules interact and create an interface in which they bury part of each domain, which obstructs access to the active site of the C-terminal domain. Upon phosphorylation at Asp56, located in the N-terminal domain very close to the interface region, the protein undergoes a conformational change that restores access to the active site (Jurica & Stoddard, 1998).

## 2.5. CheR and C-terminal pentapeptides of chemoreceptors

CheR is a methyltransferase that binds to a specific sequence of the cytosolic region of chemoreceptors. However, methylation does not occur near this specific binding site. The consensus binding sequence comprises the following amino acids: Asn-Trp-Glu-Thr-Phe (NWETF); and has been given the name of chemoreceptor pentapeptide. CheR binds the pentapeptide through a small, folded motif which consists of three  $\beta$  strands and a single  $\alpha$ -helix, called  $\beta$  subdomain (Djordjevic & Stock, 1998).

Not all chemoreceptors contain a pentapeptide. Out of the five chemoreceptors in *E. coli*, only Tsr and Tar contain a pentapeptide. However, all chemoreceptors are substrate of methylation by CheR. Therefore, it has been proposed that CheR binds to specific chemoreceptors that contain a pentapeptide and methylates the rest of chemoreceptors tethered from that position. Mutations in the pentapeptides of Tsr and Tar abolished chemotaxis to their ligands (Le Moual et al., 1997; Li et al.,

1997; Li & Hazelbauer, 2006; Okumura et al., 1998). However, the presence of pentapeptide is not an essential feature for chemotaxis, as there are many chemoreceptors lacking pentapeptide that mediate strong chemotactic responses in many bacteria (Compton et al., 2018; Corral-Lugo et al., 2018; Luu et al., 2015; Martín-Mora et al., 2019; Matilla & Krell, 2017).

When looking at the chemoreceptor repertoire of *P. aeruginosa*, only one out of 26 chemoreceptors contain a pentapeptide, the McpB/Aer chemoreceptor (García-Fontana et al., 2014). Unlike *E. coli*, *P. aeruginosa* possess more than one signaling pathway homologous to chemotaxis. These pathways are called chemosensory systems and will be discussed in detail in later sections. Having multiple systems implies that not all chemoreceptors signal through the same chemotaxis proteins, but they display specificity towards distinct paralogues. Of the four CheR paralogues present in *P. aeruginosa*, only CheR2 was found to bind the pentapeptide of McpB. Interestingly, CheR2 is located in the same operon as McpB. Moreover, CheR2 was found to have a three-amino acid insertion (GXX; GPN in *P. aeruginosa*) essential for pentapeptide recognition (García-Fontana et al., 2014). Following this rationale, CheRs can be categorized into different families based on pentapeptide binding: pentapeptide dependent CheRs (PDCheR) and pentapeptide independent CheRs (PICheR). Several studies have shown differences at the structural level between PDCheR and PICheR. The latter are thought to interact directly with the methylation site and methylate it, although the mechanism remains unsolved (Batra et al., 2016).

## 2.6. CheW and CheV

To assemble the chemoreceptors into trimers of receptor dimers, CheA needs to interact with the chemoreceptors and with a coupling protein, CheW or CheV (Abedrabbo et al., 2017). CheW is the canonical chemotaxis coupling protein and it has two subdomains. In contrast, CheV is a hybrid protein containing an N-terminal CheW-like domain fused to a C-terminal receiver domain that can be phosphorylated (Alexander et al., 2010). Moreover, CheW is more widely distributed than CheV across genomes of completely sequenced bacteria and archaea (Alexander et al., 2010). Interestingly, CheW and CheV also differ in genomic location. While CheW is usually located within a chemotaxis operon and near a CheA, CheV is more frequently found scattered across the genome (Wuichet et al., 2007).

CheW and CheV have been extensively studied at a functional level, but only CheW has been structurally resolved, both in an isolated manner or in complex with other chemotaxis proteins like CheA and MCPs. Most structural work has been performed in the model organism *E. coli* and the thermophilic bacteria *T. maritima* (Briegel et al., 2012; Falke & Piasta, 2014; Griswold et al., 2002; Li et al., 2013; Li et al., 2007; Park et al., 2006). *T. maritima* CheW is composed of two five-stranded  $\beta$

sheet domains, named subdomain 1 and subdomain 2, and two  $\alpha$ -helices (Griswold et al., 2002; Griswold & Dahlquist, 2002). *E. coli* CheW is highly similar, but it has some differences. For instance, the C-terminal helix is longer (Li et al., 2007), which contracts the active binding site with CheA, probably affecting the docking affinity between CheW and CheA. When analyzing a large number of sequences belonging to CheW from different bacteria, only three residues are conserved: Gly57, Arg62 and Gly63. In *E. coli*, many mutational analyses have determined how each one of these residues are essential for chemotaxis, as they are important for protein-protein interaction between CheA and the MCPs (Boukhvalova et al., 2002a; Boukhvalova et al., 2002b; Liu & Parkinson, 1989).

While CheW is fundamentally a coupling protein and it is essential for chemotaxis array formation (Briegel et al., 2012), CheV has an additional role. As mentioned before, the main difference between CheW and CheV is the presence of an additional REC domain in CheV. This REC domain is homologous to other response regulators that contain the conserved aspartate substrate of phosphorylation. Presumably, CheA is responsible for CheV phosphorylation, and work done in *B. subtilis* suggests that it may function as a phosphate sink to reset the chemotaxis system to its pre-stimulus state (Karatan et al., 2001; Rosario et al., 1994). Recently, a study revealed that bacteria encoding CheVs in their genome contained about four times more MCPs than bacteria without CheVs (Alexander & Zhulin, 2007). More research is needed to uncover the function of CheVs in addition to its coupling protein role.

## **2.7. CheZ, CheC, CheX and FliY**

CheZ is a phosphatase which eliminates the phosphate group from CheY-P. CheZ functions as a dimer (Blat & Eisenbach, 1996b), and it is thought that its Gln147 inserts into the CheY active site and orientates a water molecule for the nucleophilic attack (Zhao et al., 2002). CheZ is responsible for signal termination, allowing the chemotaxis system to be restored to pre-stimulus levels (Blat & Eisenbach, 1996a). Not all bacteria have CheZ in their genome. In fact, it seems to be a feature limited to the  $\gamma$ -proteobacteria family (Szurmant & Ordal, 2004). Therefore, in many other bacteria, signal termination is dealt with in alternative ways. For instance, many bacteria possess more than one CheY response regulator, where one interacts with the flagella motor and the other functions as a phosphate sink (Sourjik & Schmitt, 1998).

As the investigation in chemotaxis progressed and was broadened to other bacteria, another class of phosphatases were discovered, the CheC-type phosphatases. The CheC-type family comprises proteins like CheC, FliY and CheX, which were found to dephosphorylate CheY-P (Muff & Ordal, 2007). Interestingly, the CheC family is much more widespread than CheZ (Szurmant & Ordal, 2004). It can be found in Firmicutes and Thermotogales, and though the FliY is mainly found in these two families,

CheC and CheX can also be found in Proteobacteria, and CheX homologues are present in Spirochetes.

Structurally, the three proteins that comprise this family are quite diverse. For instance, while CheX and CheC are single domain proteins, FliY is a multidomain protein in which the C-terminus is homologous to the FliN flagellar protein of *E. coli* (Toker & Macnab, 1997). Nevertheless, sequence identity among the three proteins is always found in the consensus phosphatase active site sequence D/S-X<sub>3</sub>-E-X<sub>2</sub>-N-X<sub>22</sub>-P (Park et al., 2004). Another difference is that CheC and FliY have two active sites, while CheX has only one. Interestingly, some proteins have been found to have a CheC domain at C-terminal fused to a CheY-like receiver domain, like *Psyr\_0886* from *P. syringae*. In this case, it might not function as a canonical CheC, it could alternatively function as a phosphatase controlled by a kinase, as a phosphatase sink, or even have a novel function (Muff & Ordal, 2008).

## 2.8. CheD

CheD is a deamidase that targets specific glutamine residues present in chemoreceptors (Kristich & Ordal, 2002; Szurmant & Ordal, 2004). This process has been mostly studied in *B. subtilis*, where chemotaxis is different than that of *E. coli*. In fact, even though most bacteria contain CheD, this protein is absent in *E. coli*. Deamidation causes activation of chemoreceptors, causing an increase in CheA activity (Chao et al., 2006; Glekas et al., 2012; Szurmant & Ordal, 2004). Moreover, CheD binds to CheC and increases its phosphatase activity, functioning together in a feedback loop with CheY-P, constituting an adaptation system in *B. subtilis* (Rao et al., 2008).

CheD has been very little studied in chemotactic bacteria other than *B. subtilis*. Recently, some work has been done on its role in the regulation of the Aer2 chemoreceptor from *P. aeruginosa* PAO1 (Orillard & Watts, 2021). This strain, as other *Pseudomonas*, lack CheC and CheX, meaning that the feedback loop mentioned earlier is absent. In contrast, CheD interacts with a CheY homologue not involved in flagellar rotation, and it is thought to sequester CheD when Aer2 is actively perceiving O<sub>2</sub>. Since CheD has been seen to increase the methylation rate of Aer2 through CheR proteins, it is thought that when Aer2 is inactive, CheD is liberated to increase receptor methylation, enhancing the probability of Aer2 signaling (Orillard & Watts, 2021).

## 2.9. Chemotaxis array architecture and its diversity

As mentioned briefly in previous sections, MCPs interact with CheA and CheW to assemble into supramolecular structures called chemotaxis arrays. Chemoreceptors associate into trimers of dimers through the interaction at their cytoplasmic tips (Ames et al., 2002; Kim et al., 1999; Parkinson et al.,

2005; Studdert & Parkinson, 2004, 2005), and together with CheA and CheW, assemble into a large hexagonally packed lattice arrangement (Briegel et al., 2008, 2011, 2012; Liu et al., 2012). In *E. coli*, each signaling unit, which can be defined as the smallest structure that supports chemotactic signaling, contains 6 chemoreceptors dimers associated into trimers (trimer of dimers), 1 dimeric CheA, and 2 monomeric CheW (Figure 9) (Erbse & Falke, 2009; Kim et al., 1999; Li & Hazelbauer, 2004; Studdert & Parkinson, 2004). Cryo-electron tomography (cryo-ET) has revealed this to be a common structure for all chemotactic bacteria studied to date (Briegel et al., 2012, 2014), although some variation in terms of which proteins intervene or their stoichiometry has been reported (Yang & Briegel, 2020).

Array structure is dependent on strict regulation of the stoichiometry ratio of chemoreceptors, kinases and coupling proteins. Overexpression of one component can lead to the formation of alternative array structures (Briegel et al., 2014). Chemoreceptor arrays can even form in the absence of baseplate components, where the cytoplasmic tips of MCPs interact and they organize in a micelle-like structure or “zippers”. Moreover, modified chemoreceptors lacking their transmembrane domain can also form arrays in the presence of CheA and CheW, shaped in a “sandwich” form. However, this “sandwich” assembly has only been seen *in vitro* in the presence of crowding agents (Briegel et al., 2014; Haglin et al., 2017).

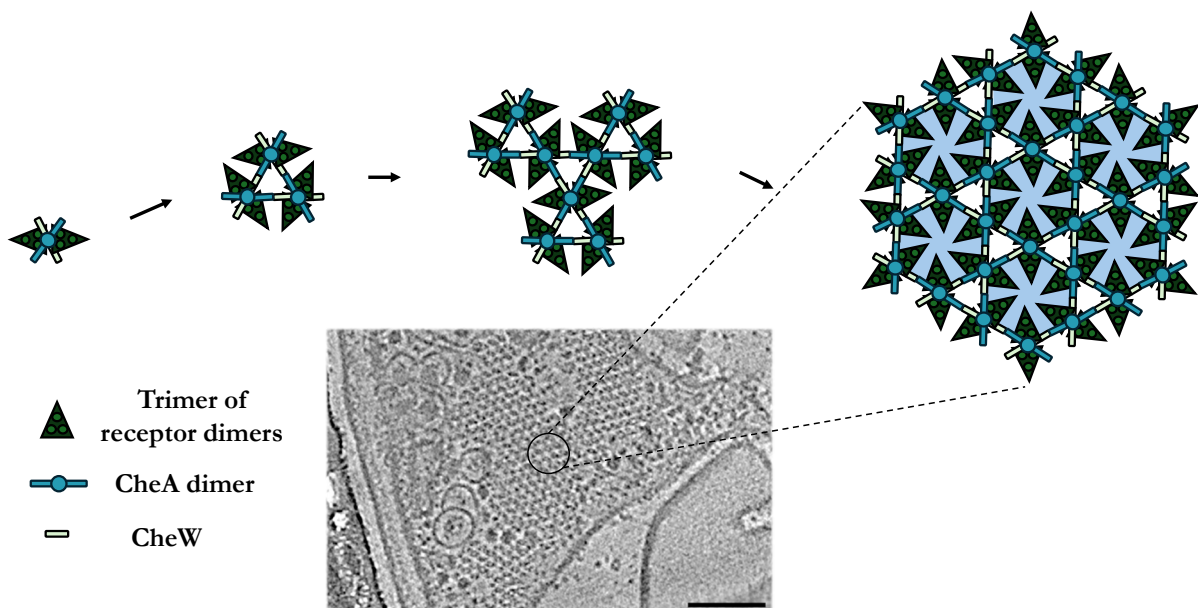


**Figure 9. Schematic formation of the hexagonally packed trimers of dimers structure.** Here it is shown the sequential assembly of the core functional unit (two trimers of receptor dimers, one CheA dimer, and two CheW monomers) forming from an individual signaling unit, and subsequently coalescing into individual hexagons, which in turn assemble into the extended superlattice. Empty hexagons without associated CheA are colored blue. Adapted from Briegel et al. (2014). Also, a tomographic slice through an array patch at the level of the chemoreceptors is shown from Briegel et al. (2014).

Chemoreceptors trimers of dimers arrange into a hexagonal pattern spaced approximately 12 nm (Briegel et al., 2009). From a side view, arrays can be seen as a continuous layer parallel to the inner membrane. The chemoreceptors can be distinguished as pillar-like densities that extend between the inner membrane and a continuous density which corresponds to the CheA-CheW baseplate (Figure 10). The side view allows distinguishing the length of the cytoplasmic region of chemoreceptors that form the array, which is an important feature for recognizing which chemoreceptors take part in the formation of the array (Briegel et al., 2009).

Some chemoreceptors naturally do not have a transmembrane domain. In fact, 14% of all chemoreceptors are predicted to be cytoplasmic (Collins et al., 2014). Cytoplasmic arrays have been observed in *V. cholerae* (Figure 10), *Rhodobacter sphaeroides*, and the archaeon *Methanoregula formicica* (Briegel et al., 2014; Briegel et al., 2015). The native structure of these arrays is fairly similar to the “sandwich” organization observed *in vitro*. Two hexagonal lattices of receptor trimers of dimers bind head-to-head and sandwich between two CheA:CheW baseplates (Yang & Briegel, 2020) (Figure 10).

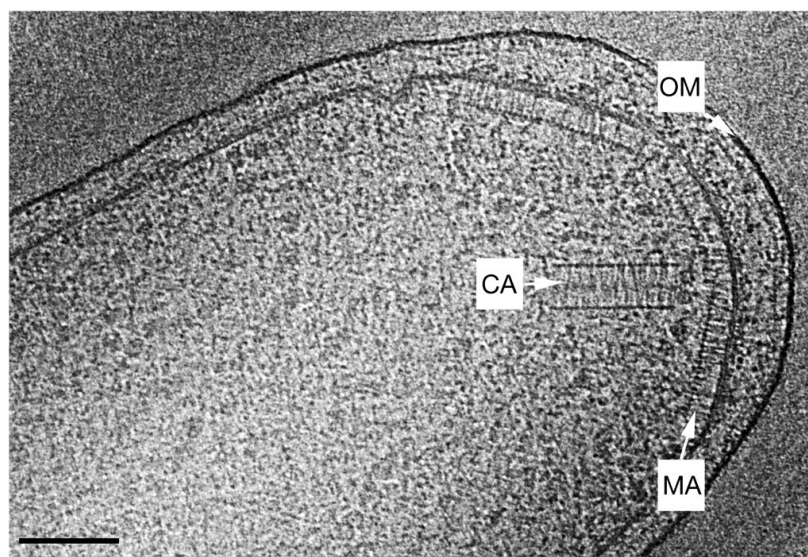


**Figure 10. Electron tomography of membrane-bound (MA) and cytoplasmic (CA) chemotaxis arrays of *Vibrio cholerae*.** OM, outer membrane. Scale bar: 100 nm). Adapted from Briegel et al. (2016).

Despite the diversity exhibited in bacteria regarding chemoreceptors and other chemotaxis proteins, a hexagonal order of chemotaxis arrays is conserved across all chemotactic bacteria. Even in archaea this pattern of organization is also conserved (Briegel et al., 2009, 2015). This strongly suggests that chemotaxis arrays have evolved into optimized structures that facilitate cellular responses. The arrays act as a foundation for receiving inputs from multiple receptors simultaneously, ensuring efficient communication with the cytoplasmic components of the chemotaxis pathway (Yang & Briegel, 2020). Moreover, the conserved array structure is thought to be essential for chemotactic cooperativity and

signal amplification through allosteric interactions within the arrays (Li & Hazelbauer, 2014; Piñas et al., 2016).

MCPs, CheA and CheW are not the only proteins who can take part in a chemotaxis array. As mentioned in previous sections, CheV is also a coupling protein found in many bacterial genomes which also contains a response regulator domain. CheV can integrate into the baseplate of the arrays and interact directly with CheA and chemoreceptors (Alexander et al., 2010). In *V. cholerae*, the protein ParP also integrates into the baseplate. ParP has a domain topologically very similar to the P5 domain of CheA and CheW, which facilitates its integration (Alvarado et al., 2017). Moreover, ParP allows localization of the chemotaxis array to the flagellar pole of the bacterium, aided by ParC (Ringgaard et al., 2018). Making sure that the array localizes near flagella could be particularly important for those bacteria that display a highly localized population of CheY proteins (Jones & Armitage, 2015). This enlightens how certain proteins of the baseplate do not only serve as structural or signaling features, but also take part in regulating its location inside the cell.

The stoichiometry of array components is also quite diverse among bacterial species. In *E. coli*, the baseplate has a ratio of monomeric CheA and monomeric CheW that varies between 1:1 and 1:2, which is a high ratio of CheA in comparison to other bacteria (Cassidy et al., 2015). *B. subtilis* displays a ratio of baseplate components for CheA:CheW:CheV of 1:1:3 (Cannistraro et al., 2011). In one of the chemotaxis arrays of *V. cholerae* the ratio for CheA:CheW:CheV is 1:7:2 (Yang et al., 2018). Furthermore, as previously mentioned, even ParP proteins can integrate into the arrays in *V. cholerae*, which illustrates how the presence of kinase is significantly lower than that of coupling proteins. When looking at the ratio of dimeric MCPs and dimeric CheA, the presence of kinase appears even lower. In *B. subtilis*, this ratio is 23:1, which is about four times higher than in *E. coli*, in which the ratio is 6:1. This shows how many chemoreceptors in a single array bind solely to the coupling protein, even if they all signal to the same kinase (Yang & Briegel, 2020). This raises the question of whether a signaling unit is always formed by 6 receptor trimers of dimers, 1 dimeric CheA and 2 monomeric CheW, as stated previously. In bacteria with low CheA abundance, this does not seem to be the case. In these bacteria, a higher cooperativity among chemoreceptors is likely needed. While one receptor trimer of dimer of *E. coli* is linked to one CheA, about 6 receptor trimers of dimers of *V. cholerae* are assigned to one CheA, based on the stoichiometry data (Yang et al., 2018). Inevitably, the signaling of one chemoreceptor must propagate horizontally to stimulate its nearest CheA.

Arrays form spontaneously and tend to associate with already existing clusters (Mauriello et al., 2018), which typically results in large arrays located at cell poles (Kentner & Sourjik, 2006). Many different chemoreceptors take part in the same array. However, not all chemoreceptors of a bacterial strain are

present in the same array. In the case of membrane-bound arrays, the length of chemoreceptors determines which ones will take part in the same array (Alexander & Zhulin, 2007; Briegel et al., 2009). “Length of chemoreceptors” refers to the physical length of the cytoplasmic region of chemoreceptors, including the number of heptad repeats in the signaling domain, the number of HAMP domains, the presence or absence of PAS domains, and additional linker regions (Möglich et al., 2009; Parkinson, 2010). As mentioned in earlier sections, chemoreceptors can be classified into different classes depending on the number of heptads, inherently the length of chemoreceptors. All five chemoreceptors of *E. coli* are of 36H class, and they cluster together in a single array. However, experimental evidence showed how artificially shortened chemoreceptors expressed in *E. coli* assembled into a separated, different chemotaxis array (Herrera Seitz et al., 2014). In other bacteria that possess many chemoreceptors of different lengths, several chemotaxis arrays can be found separately. This is the case of *V. cholerae*, where two distinct arrays are positioned at the membrane, and one is cytoplasmic (Ortega et al., 2020) (Figure 10). Chemotaxis arrays positioned at the membrane seem to be stricter when it comes to which chemoreceptor can take part in the array. In contrast, in the cytoplasmic array of *V. cholerae*, there are chemoreceptors of 44H class that assemble with chemoreceptors of 20H class (Briegel et al., 2014; Briegel et al., 2016).

### 3. Energy taxis

Energy taxis can be defined as changes in motility patterns in response to internal metabolic alterations (Schweinitzer & Josenhans, 2010). Bacteria would use energy taxis to navigate towards niches that are metabolically favorable. Initially, the definition of chemotaxis was restricted to changes in motility in response to external chemoeffectors that do not need to be transported or metabolized (Adler, 1969). These two processes have recently been studied jointly, as many MCPs have been described to directly sense metabolizable compounds, which influence bacterial metabolism.

A wide variety of compounds can elicit energy taxis; first, metabolizable compounds such as sugars, amino acids, or organic acids; second, compounds that serve as electron acceptors, like oxygen, nitrate, fumarate, or DMSO; and third, compounds that generally affect bacterial metabolism, like non-metabolizable substrate analogs or metabolic inhibitors (Alexandre et al., 2004; Baraquet et al., 2009; Taylor & Zhulin, 1999) (Figure 11). In contrast to chemotaxis, where the chemoeffectors are sensed directly by MCPs or together with an SBP, stimulation of energy taxis is the consequence of metabolizing those elicitors. Therefore, energy taxis effectors need to be in higher concentrations than chemotaxis effectors. This is the main difference with chemotaxis, in which metabolizable compounds can be sensed directly by specific MCPs at very low concentrations and mediate taxis towards them.

Energy sensing connected to taxis has been reported in many bacteria, which is not surprising as energy generation is an essential process for bacterial survival. Energy generation differs greatly among bacteria, as it depends on their natural habitat and their metabolic requirements. Four major types of energy taxis mediated by MCP-like sensor proteins can be described: Phototrophic energy taxis, PAS-domain MCP energy taxis sensors, Proton Motive Force (PMF) sensing MCPs and Hem-AT-like MCP aerotaxis sensors.

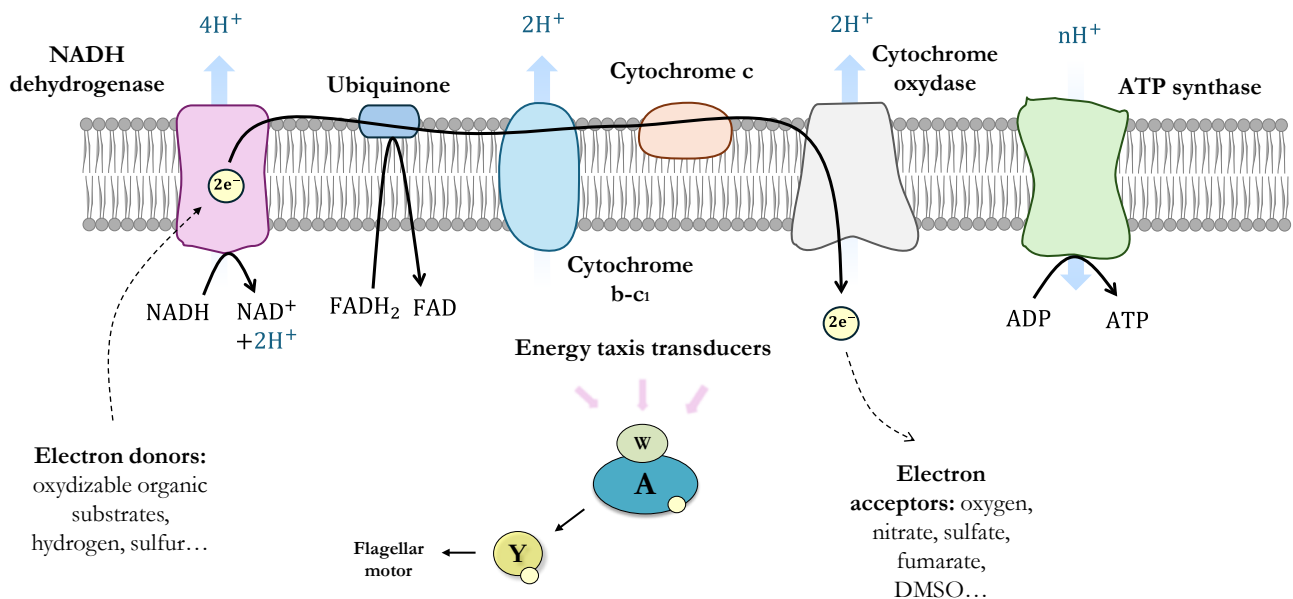


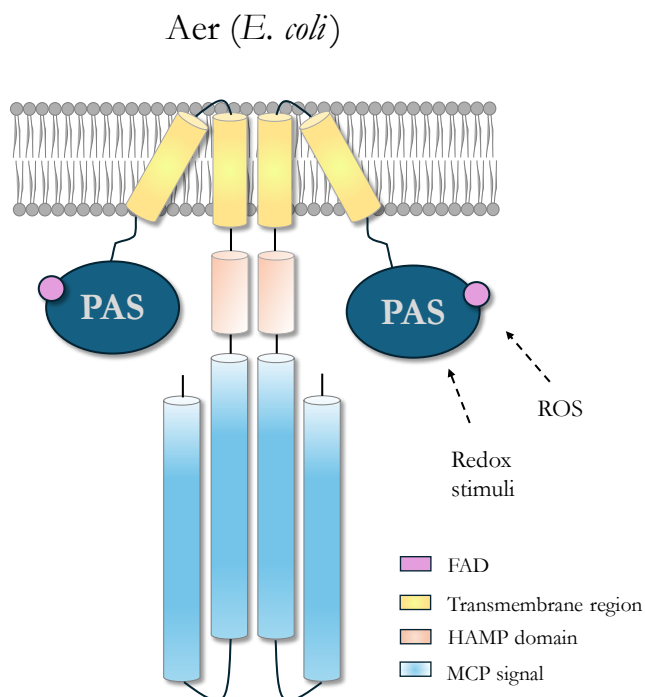
**Figure 11. Energy taxis system.** Energy taxis transducers receive information from the electron transport system and/or proton motive force. Changes in the concentration of electron donors or acceptors trigger the signaling cascade. Small light-yellow circles represent phosphate groups. Adapted from Alexandre et al. (2004). NADH, Nicotinamide Adenine Dinucleotide; FAD, Flavin Adenine Dinucleotide; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DMSO, dimethyl sulfoxide.

### 3.1. Phototrophic energy taxis

Phototaxis was one of the earliest discoveries related to energy taxis (Alam et al., 1989; Spudich et al., 1986; Yao & Spudich, 1992). Light is the most important source of energy for many environmental bacteria. Light or photons are sensed by specific proteins that contain a photosensory domain. Two of the most well-known photosensory domains are the LOV (light, oxygen, voltage), which belong to the PAS domain superfamily; and PHY (phytochrome-like) photosensors (Losi & Gärtner, 2008), which contain a GAF domain. Photoreceptor proteins do not usually take part in MCPs (Yao & Spudich, 1992), although a few cases have been reported so far of photosensors that are fused to an MCP-like output domain (Losi & Gärtner, 2008; Ng et al., 2003).

### 3.2. PAS-domain MCP energy taxis sensors

Perception of stimuli provided by the electron transport chain is a suitable mechanism for bacteria to assess their metabolic state. The best characterized energy taxis sensor linked to the electron transport is the Aer chemoreceptor from *E. coli* (Figure 12). This MCP contains a PAS domain as ligand binding domain, but in contrast to the canonical MCP structure, this is not located in the periplasm, but rather facing the cytoplasm. The PAS domain is linked to the membrane by an anchor of hydrophobic amino acids (Amin et al., 2006; Taylor, 2007). PAS domains, as mentioned in previous sections, are widely distributed in all kingdoms of life which can sense light, oxygen, or redox potential as input signals (Taylor, 2007). Aer function is mediated by binding FAD and guides bacteria in a gradient of rapidly oxidizable substrates (Greer-Phillips et al., 2003). Edwards et al., 2006 determined how Aer-dependent taxis was linked to redox changes in the cell. Similarly to a canonical MCP, Aer also contains a HAMP domain which transduces the signal downstream, probably through a direct interaction with the PAS domain (Taylor, 2007; Watts et al., 2008). Aer has been observed to take part in the chemotaxis array of *E. coli*, although it does not need other chemoreceptors in the array to support energy taxis (Hazelbauer et al., 2008).



Besides Aer from *E. coli*, many other chemoreceptors containing PAS domains have been described: *V. cholerae* Aer-1, Aer-2 and Aer-3 (Boin & Häse, 2007), *P. aeruginosa* Aer and Aer-2 (Hong et al., 2004), *Ralstonia solanacearum* Aer1 and Aer2 (Jian & Caitilyn, 2006), *Azospirillum brasilense* AerC (Xie et al., 2010), *P. chlororaphis* PCL1606 Aer1-1 and Aer1-2 (Arrebola & Cazorla, 2020), and *P. syringae* pv. tabaci AerB and AerC (Tumewu et al., 2022).

**Figure 12. Schematic representation of the *E. coli* Aer chemoreceptor.** ROS, reactive oxygen species. Adapted from Schweinitzer & Josenhans (2010).

### 3.3. Proton Motive Force (PMF) sensing MCPs

These aerotaxis sensors are structurally and functionally different from PAS domain containing MCPs. They exhibit a canonical MCP structure with a periplasmic LBD, a transmembrane region, and a conserved signaling domain. The prime example of these kind of energy taxis sensors is the serine chemoreceptor from *E. coli* Tsr (Greer-Phillips et al., 2003). Apart from serine, Tsr mediates taxis responses to oxygen (Rebbapragada et al., 1997), carbon sources (Greer-Phillips et al., 2003), pH (Umemura et al., 2002), and temperature (L. Lee et al., 1988). Since Tsr responses to carbon sources were observed to be directly correlated with their suitability for growth (Greer-Phillips et al., 2003), and it also mediates taxis to an electron acceptor (oxygen), it was hypothesized that Tsr could be a PMF sensor, which can be seen as the outcome of energy generation. A link between the Tsr-mediated taxis and the PMF has been experimentally proven by Edwards et al. (2006). It is thought that PMF could be sensed by charged amino acids present in the MCP signaling domain. However, no information regarding these specific amino acids has been obtained. Although there are many homologues to *E. coli* Tsr in other bacteria, only a few examples of this kind of chemoreceptors have been observed to be involved in energy taxis so far. AerA from *P. syringae* pv. tabaci, homologous to *E. coli* Tsr, has been observed to be involved in oxygen perception through mutational analysis (Tumewu et al., 2022).

### 3.4. Hem-AT-like MCP aerotaxis sensors

This kind of chemoreceptors perceive haeme-bound oxygen directly. Strictly speaking they should not be categorized as energy taxis sensors, as they bind their ligand directly. However, since oxygen is the most important molecule for aerobic metabolism, they are included as energy taxis transducers. Hem-AT-like MCPs were first identified in *D. vulgaris* (Fu et al., 1994). Other examples can be found in bacteria from the *Bacillus* and *Halobacterium* genus, reported to mediate aerotaxis (Hou et al., 2000). These chemoreceptors lead bacteria to their preferred oxygen concentration. However, additional roles to aerotaxis have been recently discovered for this type of chemoreceptors. A recent study shows how the HemAT MCP from *B. subtilis* directly binds and mediates taxis to ethanol, although the heme group is not involved in its binding (Tohidifar et al., 2020).

## 4. Chemosensory systems

Chemotaxis is arguably the most complex signaling pathway present in bacteria, which has been explained in detail in previous sections. Most sequenced bacterial genomes contain genes homologous to those that control chemotaxis in *E. coli* or *S. enterica*, where chemotaxis has been best studied. These genes are usually accompanied by the presence of genes that encode flagellar proteins.

Therefore, chemotaxis and motility genes are widespread in bacteria and have been selected along evolution probably because their presence results beneficial to thrive in different environments. However, not all chemotaxis genes have been shown to be involved in modulating flagellar motility, which is why the term “chemosensory system” is more adequate when referring to signaling pathways that are homologous to chemotaxis but could be regulating other processes. Some experimental evidence is available on chemosensory systems controlling processes like type IV pili (Tfp) motility (Bhaya et al., 2001; Zusman et al., 2007) and other cellular functions, such as development (Berleman & Bauer, 2005b; Kirby & Zusman, 2003a), second messenger levels (O’Connor et al., 2012), biofilm formation (Heering & Ringgaard, 2016), cell morphology (Bible et al., 2008), cell-cell interactions, and flagella biosynthesis (Berleman & Bauer, 2005a).

In addition to the canonical chemotaxis proteins, chemosensory systems can contain proteins like CheC or CheX, which are absent in *E. coli*; or even lack some proteins that are present in *E. coli*, like CheZ, CheB and CheR. Experimental and phylogenomic data have led to the classification of the diverse chemosensory systems in three main functional groups: those that evolve to control flagellar motility (F), type IV pilus-based motility (Tfp), and alternative (non-motility) cellular functions (ACF) like the control of levels of second messengers (Wuichet & Zhulin, 2010). The F group is the most diverse, containing seventeen classes (F1-F17), while the other two groups contain a single system class. All chemosensory systems include the essential components constituting the core of the pathway: MCPs, kinase, adaptor protein, and a response regulator. Diversity within a group is mainly due to the variety of auxiliary proteins that accompany the core of the system and the functional nature of the response regulator (Wuichet & Zhulin, 2010).

It is not unusual to find genes encoding for different chemosensory systems within the same bacterium, suggesting a versatile ability of response through different specialized pathways (Porter et al., 2011). In those bacteria, experimental evidence has shown that usually one chemosensory system takes part in chemotaxis, while the other chemosensory systems might have other roles in cellular physiology (He & Bauer, 2014). These studies highlight the diversity of chemosensory systems among different bacteria and their broad range of roles as a response to chemoperception (Bardy et al., 2017; Colin & Sourjik, 2017).

It is important to note that chemotaxis arrays also form in the case of those chemosensory systems that might not be involved strictly in chemotaxis. Until now we named this structure “chemotaxis array”, but it can also be named “chemosensory array”, and from now on both terms will be used to describe these structures.

#### 4.1. Functional variety of chemosensory systems

Chemosensory systems are very diverse when it comes to their functions. As mentioned earlier, chemosensory systems are organized in F, ACF, and Tfp categories depending on phylogeny and functionality (Wuichet & Zhulin, 2010). Within the F systems we find 17 different classes (F1-F17), some of which are exemplified in Figure 13.

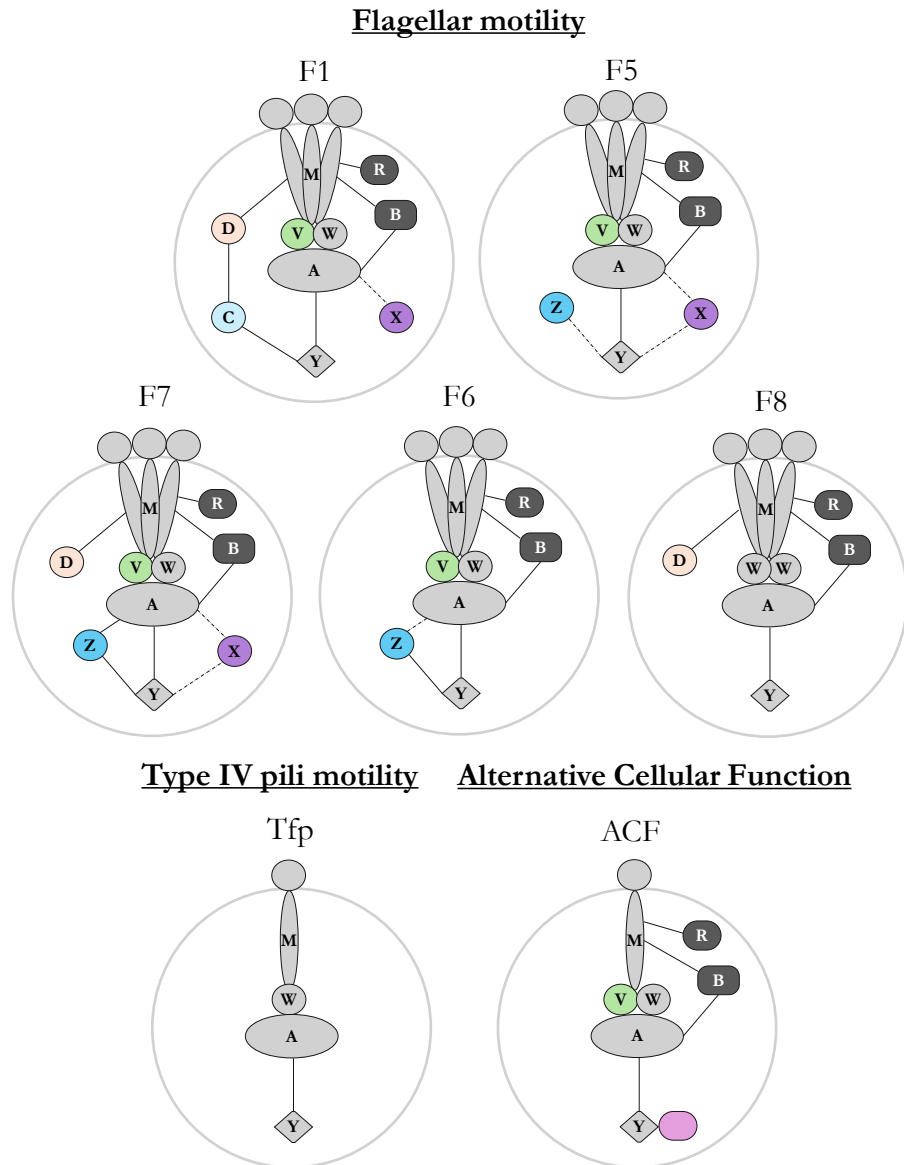


**Figure 13. Some of the major classes of chemosensory systems.** Typical architectures are displayed, but protein repertoire within each class may vary. Tfp and ACF pathways typically contain a single MCP, whereas multiple MCPs are associated with all other classes. Core essential components (M, MCP; A, CheA; W, CheW; Y, CheY) are in light grey; core dispensable components (R, CheR; B, CheB) are in dark grey; the auxiliary proteins (D, CheD; V, CheV; C, Z and X, CheC, CheZ and CheX) and output domain of response regulator protein from ACF system are shown in color. Experimentally confirmed protein-protein interactions are represented by solid lines; dashed lines indicate predicted interactions. Adapted from Gumerov et al. (2021).

Notably, even though there is a classification, their category does not always reflect their output function. For instance, the classic chemotaxis system of *E. coli* is categorized as F7, and the chemosensory system responsible for the same exact process in *B. subtilis* is categorized as F1. Even when the output of these two pathways is exactly the same, both chemosensory systems work in the opposite way. While attractant binding leads to CheA inactivation in *E. coli* (Parkinson et al., 2015), it leads to CheA activation in *B. subtilis* (Rao et al., 2008). Therefore, even though their output is to modulate flagellar rotation, they belong to different classes because their mechanism of action is different.

Some bacteria encode several chemosensory systems that are categorized as F, and therefore, putatively involved in flagellar motility. In this case, usually both chemosensory systems regulate different aspects of this process. For instance, the F7 and F5 chemosensory systems of *A. brasilense* control two different features of flagellar motility: motor reversal frequency and transient increases in swimming speed (O’Neal et al., 2019). Impressively, *R. sphaeroides* contains two F7 and one F8 systems, and two of them (one F7 and F8) work collectively to control the stop frequency and duration of a single subpolar flagellum rotation (Beyer et al., 2019), while the remaining F7 system controls chemotaxis mediated by several polar flagella (Hernández-Valle et al., 2017). *P. aeruginosa* has two F systems, one F6 and one F7. The F6 system controls chemotaxis (Ortega et al., 2017), while the F7 system is involved in regulation of oxygen stress response (Orillard & Watts, 2021).

Tfp systems putatively control twitching motility, driven by the type IV pili, which allows bacteria to move across solid surfaces. The best studied Tfp system is that of *P. aeruginosa* (Inclan et al., 2016), but it has also been described in other bacteria belonging to the  $\gamma$ -proteobacteria family (Corral et al., 2020; Corral et al., 2020; Cursino et al., 2011). However, Tfp systems have also been shown to be involved in traits unrelated to surface motility. This is the case for Tfp systems of *Synechocystis* sp. PCC 6803 and *Nostoc punctiforme*, which control phototaxis but also developmental and biosynthesis processes (Campbell et al., 2015; Yoshihara et al., 2002). Remarkably, the chemosensory systems involved in regulating twitching motility in *Myxococcus xanthus* are called Frz and Dif, and they are not categorized as Tfp, but as ACF and F1, respectively (Mauriello et al., 2018).

ACF systems are involved in processes that, in principle, are unrelated to motility. For instance, the ACF system of *Rhodospirillum centenum* regulates the formation of stress-resistance structures (He & Bauer, 2014). In *A. brasilense*, the ACF system is responsible for flocculation in liquid cultures (Bible et al., 2015). Remarkably, *M. xanthus* contains four chemosensory systems annotated as ACF. Three of them associate to form a large chemosensory module that controls fruiting body formation (Moine et al., 2014), while the remaining system regulates the expression of key genes involved in this process (Kirby & Zusman, 2003b; Willett et al., 2013). One of the best studied ACF systems is that of *P.*

*aeruginosa*, also called the Wsp system, which controls biofilm formation through modification of intracellular c-di-GMP levels (Huangyutitham et al., 2013). Interestingly, many chemosensory systems from different categories have emerged to regulate biofilm related traits. For example, the Flm system in *Comamonas testosteroni*, categorized as Tfp, regulates biofilm formation (Huang et al., 2019), as well as the F5 and F7 systems from *C. crescentus*, who also appear to be involved in this process (Berne & Brun, 2019). In the plant-associated bacterium *Azorhizobium caulinodans*, the F5 system is important for plant colonization as well as being involved in biofilm formation (Liu et al., 2018a). Moreover, some studies have reported the possible crosstalk among different chemosensory systems to regulate biofilm formation (Berne & Brun, 2019; Huang et al., 2019).

Despite the many examples given here, the role of chemosensory systems is far from being well understood. Chemosensory systems exhibit so many variations among different bacteria and even those which are homologues can sometimes display different roles. In addition, the fact that systems categorized as F do not work in modulating chemotaxis, but other processes, shows how systems evolved to mediate other functions (Gumerov et al., 2021). This manifests how assigning a category to a chemosensory system is not a good predictor for its functionality.

## **5. Relevance of chemoperception in plant associated bacteria**

Chemoperception, covering chemotaxis, energy taxis, and additional roles of chemosensory systems in response to environmental conditions, has been established as a major mechanism to ensure a successful life cycle, as it allows bacteria to locate preferential niches to develop properly. In the case of pathogens, chemotaxis has been demonstrated to be crucial for the virulence of bacteria affecting humans, animals, and plants (Matilla & Krell, 2018, 2024). Specifically for plant pathogens, chemotaxis is particularly important to detect entry sites and ensure host colonization (Matilla & Krell, 2018). Plant openings like stomata or wounds generate gradients of compounds that bacteria can use as signals to detect and move towards those openings. Yao and Allen (2006) illustrate this very well using the global plant pathogen *R. solanacearum*. They used the wild type and mutants defective in chemotaxis and motility to infect tomato plants by spray inoculation and syringe-infiltration. They observed how these mutants only had impaired infection capacity when inoculation was done by spray, which required an active movement towards plant openings (Jian & Caitilyn, 2006). Melotto et al. (2006) also showed how bacteria perform chemotaxis towards open but not closed stomata. Antúnez-Lamas et al. (2009) also showed how *Dickeya dadantii* 3937 (Dd3937) was able to move towards wounds in chicory leaves. All these studies manifest the importance of chemotaxis for plant pathogenic bacteria.

The importance of chemotaxis in plant pathogens is also supported by the fact that phytopathogenic bacteria contain a higher number of chemoreceptors than average. While 90% of phytopathogenic bacteria contain chemotaxis-related genes (Ortega, Zhulin, & Krell, 2017), such genes are only found in 47% of all bacteria (Sanchis-López et al., 2021).

Several chemoreceptors from different bacteria have been found to be involved in virulence. For instance, the PsPto-PscA from *P. syringae* pv. tomato DC3000 and the PscB and PscC2 from *P. syringae* pv. tabaci bind amino acids and a mutation in these chemoreceptors impaired virulence in tomato and tobacco plants (Cerna-Vargas et al., 2019; Tumewu et al., 2021). The Mcp2 from *Xanthomonas oryzae* mediates chemotaxis towards amino acids and is involved in virulence in rice (Verma et al., 2018). The McpM from *Ralstonia pseudosolanacearum* mediates chemotaxis towards L-malate and was found to have a role in virulence in tomato plants (Hida et al., 2015). The Dd15070 chemoreceptor from Dd3937 binds nitrite and nitrate, and its mutation affects nitrogen metabolism and virulence in potato plants (Gálvez-Roldán et al., 2023).

Even though all compounds mentioned above are present in plants, they are also present in many other habitats and are widely used nutrients. There is scarce information regarding chemoreceptors that respond to compounds only present in plants, like phytohormones, although some compounds are known to be attractants to specific bacteria. One example is the chemoattraction of Dd3937 towards the plant hormone jasmonate (Antúnez-Lamas et al., 2009). Two candidate chemoreceptor genes have been proposed to mediate this response, but the ligand perception mechanism has not been elucidated (Río-Alvarez et al., 2014). Several experiments support that jasmonate perception is needed for the virulence of Dd3937 (Antúnez-Lamas et al., 2009; Río-Alvarez et al., 2014). Chemotaxis to other phytohormones like auxins and salicylates was observed in several plant-associated bacteria (Gavira et al., 2021; Rico-Jiménez et al., 2022).

Chemotaxis has also been found to be important in non-pathogenic plant-associated bacteria. For instance, plant-growth-promoting rhizobacteria (PGPRs) respond to root exudates and use chemotaxis to colonize the rhizosphere (Feng et al., 2021). Root exudates contain a wide variety of molecules, including amino acids, organic acids, sugars, alcohols, polyamines, fatty acids, purines, phytohormones, terpenoids, flavonoids, and benzoxazinoids (Badri & Vivanco, 2009; Pétriacq et al., 2017). Sufficient work has been done in the chemotaxis of *P. putida* KT2440, which is an efficient root colonizer. This bacterium responds to compounds present in root exudates, such as organic acids, amino acids, sugars, polyamines, GABA, or inorganic acid (Ortega, Zhulin, & Krell, 2017). Similar compounds have been identified to be subject of positive chemotactic responses of *Sinorhizobium meliloti*, the cognate symbiont for alfalfa (*Medicago sativa* L.), a very relevant crop in terms of agriculture (Compton et al., 2018). Interestingly, the commercially used PGPR *Bacillus velezensis*

SQR9 was found to be chemotactically attracted to 44 out of 98 compounds present in root exudates of cucumber plants (Feng et al., 2018).

Energy taxis also plays a crucial role in the lifestyle of plant associated bacteria. In fact, *A. brasilense* is one of the bests studied bacteria in terms of energy taxis and niche colonization (Alexandre et al., 2000). This bacterium is a free-living diazotroph and is usually found in association to plant roots (Greer-Phillips et al., 2004). It has an aerobic lifestyle and can use fructose or organic acids as growth substrates (Zhulin et al., 1996). Moreover, it can use nitrate as an alternative electron acceptor. Even though it displays an aerobic metabolism, *A. brasilense* has a preference for microaerobic environments (<1% O<sub>2</sub>), in which it can fix nitrogen (Zhulin et al., 1996). Energy taxis was found to be a dominant phenotype in *A. brasilense* because it only displayed attraction towards substrates that supported growth, indicating a strong link between taxis and metabolism (Alexandre et al., 2000). Two chemoreceptors were found to be key for energy taxis in *A. brasilense*. Tlp1, which contains no PAS domain, was the first one to be studied and it is hypothesized to be similar to *E. coli* Tsr (Greer-Phillips et al., 2003). Tlp1 was observed to be important for navigation towards preferred niches in the rhizosphere and for root colonization. AerC contains two PAS domains and mediates taxis to microaerobic environments that allow nitrogen fixation (Xie et al., 2010). Some work related to energy taxis has also been done in the global plant pathogen *R. solanacearum*. This bacterium encodes two genes similar to *E. coli* *Aer*, *Aer1* and *Aer2*. Yao and Allen (2007) determined how these two genes could restore the wild-type phenotype of an *E. coli aer* mutant, indicating their functional equivalence (Jian & Caitilyn, 2007). Moreover, *Aer1* and *Aer2* were found to be important for enabling bacteria to locate and aggregate near plant roots (Jian & Caitilyn, 2007).

## **6. Model phytopathogenic bacteria**

### **6.1. *Pseudomonas syringae* pv. tomato DC3000**

*Pseudomonas syringae* is a complex of Gram-negative aerobic bacteria with rod-shaped cells, typically of 1.5 µm long and 0.7-1.2 µm in diameter. The cells are motile using at least one polar flagellum, and their growth is optimal at temperatures ranging from 22 to 30 °C (Arnold & Preston, 2019). Many strains belonging to *P. syringae* are capable of infecting many plant species that are economically relevant. Examples are the bacterial speck of tomato, halo blight on beans, bleeding canker on horse-chestnut, or canker on kiwifruit. The strains of *P. syringae* that infect specific cultivars are called pathovars, and around 60 pathovars (pv.) have been recognized for this species. The *P. syringae* complex is currently divided into 13 phylogenetic groups, often called phylogroups, and strains belonging to the same phylogroup possess common features. For instance, strains in

phylogroup 2 secrete a reduced number of effector proteins than strains in phylogroups 1 and 3, and normally produce a toxin called syringomycin (Arnold & Preston, 2019).

Interestingly, *P. syringae* can also be found in niches unrelated to crops, such as lakes, snow-melt water, clouds, rainfall, and a lot of plants that do not represent important agricultural crops. In many cases, those strains are genetically similar to those found infecting crops and can be pathogenic (Morris et al., 2008). In the context of plant pathology, bacteria from the *P. syringae* complex are generally described as hemibiotrophic pathogens that infect local regions of aerial structures of plants, like leaves and fruits. Bacterial populations can be transmitted to other structures of the plant due to bacterial excretion or to rain splash, starting a new infection cycle (Preston, 2000). *P. syringae* exhibits two spatially and temporally differentiated stages in a successful infection cycle. First, an epiphytic stage where bacteria arrive at the surface of the plant tissue; and second, one where bacteria enter the apoplast through natural openings like stomata or wounds (Boller & Felix, 2009; Hirano & Upper, 2000; Melotto et al., 2008) and multiply aggressively if the conditions are optimal (Figure 14).

*P. syringae* pv. tomato DC3000 (PsPto) is a rifampicin-resistant derivative of *P. syringae* pv. tomato DC52, which infects tomato plants (Cuppels, 1986a). This strain emerged as a model bacterium to study plant-bacteria interactions, mainly because it can also infect the model plant species *Arabidopsis thaliana* and cause hypersensitive response (HR) on tobacco plants, two model organisms in the study of plant immunity.

Plant surfaces are generally considered a hostile environment for microbes. Interestingly, there are many pathovars within the *P. syringae* species that are very well adapted to a life on the surface of leaves. These strains have evolved strategies and mechanisms to cope with nutrient limitation and sudden changes in temperature, humidity, and sunlight (Lindow & Brandl, 2003). However, this is not the case for PsPto. Several experiments determined that PsPto is a weak epiphyte in comparison to other *P. syringae* strains. When spray inoculated on tomato leaves, if PsPto does not enter the plant apoplast in approximately 48 hours, it dies (Boureau et al., 2002). Regardless of its low epiphytic strength, PsPto can be very aggressive once inside host tissues.

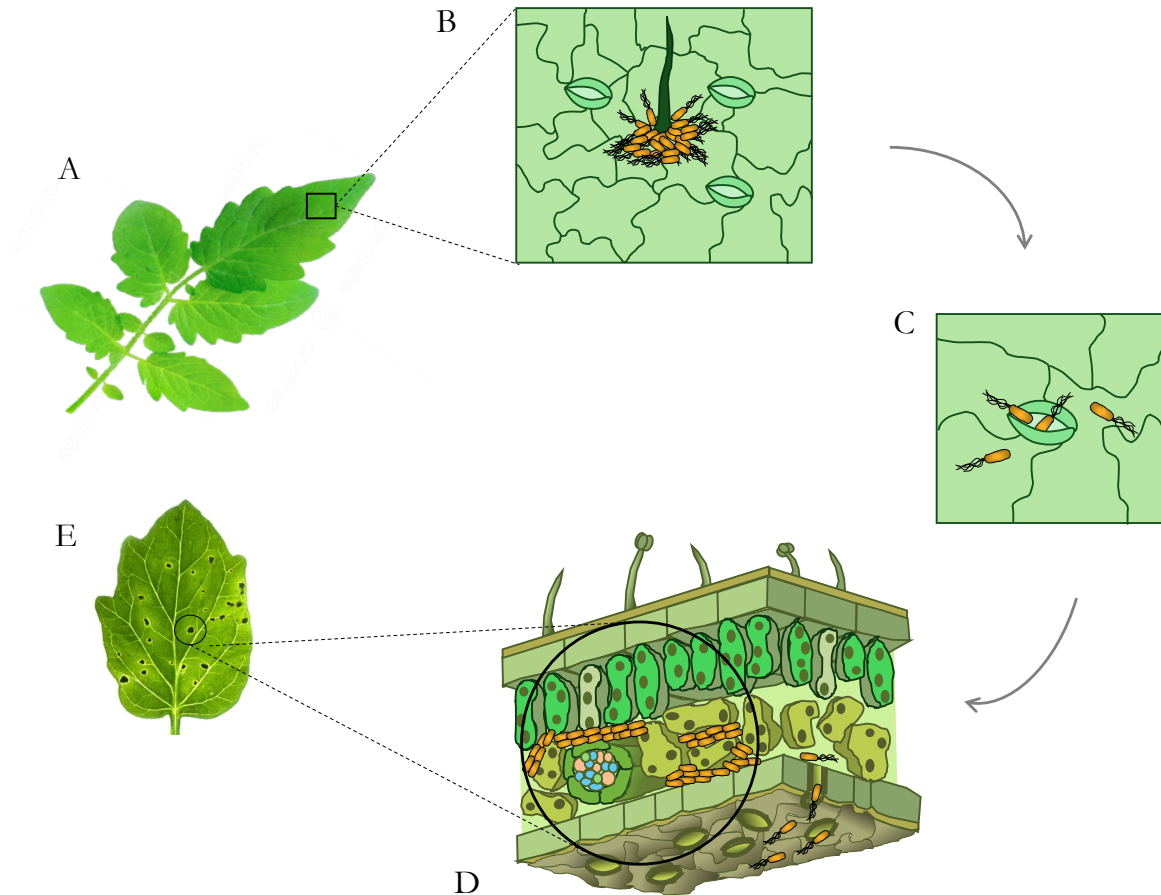


**Figure 14. A diagram of the infection cycle of *Pseudomonas syringae*.** (A) Healthy plant leaves. (B) Bacteria on the leaf surface, illustrating aggregation of some bacteria near a trichome. (C) Bacteria penetrating an open stoma. (D) A cross section of a leaf showing bacteria colonizing and multiplying in the plant apoplast. (E) Appearance of disease-associated necrosis and chlorosis. Adapted from Xin & He (2013).

*P. syringae*, as any other plant pathogen, needs to surpass the plant immune response. Briefly, the plant immune system consists of two barriers of defense, the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and the effector-triggered immunity (ETI). PTI consists of a first barrier where plants respond to the detection of PAMPs by proteins located at the plasma membrane (PM) of plant cells called PM-localized pattern recognition receptors (PRRs) (Boller & Felix, 2009; Schwessinger & Ronald, 2012). PTI is usually overcome by pathogens that have evolved to be adapted to susceptible hosts thanks to the activity of virulence factors, such as type III effectors (Block & Alfano, 2011; Boller & He, 2009; Dou & Zhou, 2012; Feng & Zhou, 2012). As a response to pathogens overcoming PTI, plants have evolved ETI, which involves recognition and neutralization of pathogen effectors by nucleotide-binding/leucine-rich-repeat (NLR) resistance proteins. Generally, PTI can be seen as a first layer of defense against general microbes, and ETI as a more specific mechanism to prevent virulent pathogens from causing diseases in their host.

Some typical immune responses are known to include callose depositions at the infection site, antibacterial phytoalexins, reactive oxygen species (ROS), pH changes, and possibly restriction of nutrient release (Xin & He, 2013). Moreover, ETI is frequently associated with the HR at the infection site, which involves programmed cell death. Plant hormones play a major role in plant immunity, and specifically in the defense strategies against *P. syringae* (Robert-Seilanianantz et al., 2011). One example is salicylic acid (SA), which needs to be accumulated in the infection site and its vicinity for full exhibition of PTI and ETI (Vlot et al., 2009). Moreover, it is crucial to develop what is called systemic acquired resistance (SAR), a mechanism that confers long-lasting protection against pathogens (Durrant & Dong, 2004). Plant hormones related to defense exhibit different effects depending on the attacking pathogen. Jasmonate (JA), for instance, is a plant hormone very well known for its role in the defense against necrotic pathogens and insects. However, it promotes *P. syringae* infection, due to the antagonistic relationship between SA and JA signaling (Browse, 2009; Glazebrook, 2005; Zheng et al., 2012).

PsPto contains one circular chromosome and two plasmids as genetic material. Approximately, 5% of the genome encodes virulence-related genes, which illustrates the potential of PsPto to be a plant pathogen (Buell et al., 2003). The main virulence factors of PsPto include protein secretion systems, and production of toxins and phytohormones (Xin & He, 2013).

PsPto encodes types I, II, III, IV, V, and VI, and twin-arginine transporter (Tat) secretion systems (Cunnac et al., 2009; Lindeberg et al., 2008), but only Tat, type II, and type III are significant when it comes to virulence. The Tat system is involved in transporting folded proteins from the cytoplasm to the periplasmic space (Palmer & Berks, 2012), and it is conserved among Gram-negative bacteria. Mutations in the Tat system cause lack of fitness and virulence in *Arabidopsis* and tomato plants. In tobacco, which is a nonhost plant, these mutations also affect the strains' ability to elicit HR (Bronstein et al., 2005; Caldelari et al., 2006). Among the proteins exported to the periplasm by Tat we can find phospholipases, amidases, and aminotransferases (Bronstein et al., 2005; Caldelari et al., 2006). The type II secretion system secretes two phospholipase C (Plc) proteins, in addition to a pectin lyase and two cellulases (Bronstein et al., 2005; Joardar et al., 2005). Its role in virulence was suggested when analyzing two mutants of the type II secretion system components, which displayed reduced chlorotic cell death in *Arabidopsis* (Bronstein et al., 2005). The type III secretion system is encoded by *hrp* (hyper-sensitive response and pathogenicity) and *hrc* (*hrp* conserved) genes. This system resembles a syringe-like supramolecular complex that is located in the membrane and across the periplasmic space. This type III secretion system is key for PsPto virulence, and it is conserved among Gram-negative pathogens, regardless of the nature of their host (Büttner & He, 2009; Galán & Collmer, 1999). The proteins secreted by this system are called type III effectors, which function in suppressing plant immunity or in promoting bacterial entry into the apoplast and fitness. *P. syringae*

pathovars possess on average more than 20 effectors, although they present a high degree of redundancy (Bundalovic-Torma et al., 2022).

PsPto produces a variety of toxins known to have a role in virulence (Bender et al., 1999). Many belong to the syringomycin group, which consist of a polar peptide head with a lipophilic tail composed of a fatty acid (Iacobellis et al., 1992). Another important example is coronatine, which might be the most studied toxin produced by *P. syringae*. Coronatine is a non-host-specific polyketide toxin composed of two structural components, coronafacic acid (CFA) and coronamic acid (CMA). This toxin functions in promoting bacterial entry into the apoplast by mimicking the plant hormone jasmonate, stimulating stomata aperture and suppressing the host defenses mediated by salicylic acid (Zheng et al., 2012).

*P. syringae* also produces phytohormones such as the auxin indole-3-acetic-acid (IAA), which has been shown to play a role in modulating disease susceptibility (Chen et al., 2007; Ding et al., 2008; Navarro et al., 2006; Robert-Seilanianantz et al., 2011).

PsPto encodes 49 predicted chemoreceptors (Ulrich & Zhulin, 2010). Out of the 49, 36 show a canonical topology, with a periplasmic LBD flanked by two TM regions, typical for sensing extracytoplasmic signals. Among them, the vast majority of chemoreceptor LBDs are of the parallel helix type (4HB, HBM, NIT, and PilJ), and only a few form the curved  $\beta$ -sheet (sCache and dCache). Nine chemoreceptors possess one or multiple PAS domains of cytosolic location that are likely to be involved in the sensing of cytosolic signals, such as the redox state or oxygen. Four other chemoreceptors are membrane bound but lack LBDs, and it is hypothesized that they respond to physicochemical stimuli like temperature or osmotic pressure. Only one chemoreceptor had been characterized for PsPto at the start of this thesis, the PsPto-PscA, a cluster II chemoreceptor belonging to class Ia. It is responsible for binding and mediating chemotaxis to D-Asp, L-Asp, and L-Glu, and it was found to play a role in the early stages of infection in tomato plants (Cerna-Vargas et al., 2019).

PsPto encodes four main chemosensory systems in independent gene clusters (Gumerov et al., 2020). One cluster has been associated with chemotaxis function, but the other chemosensory systems still remain to be characterized.

## **6.2. *Dickeya dadantii* 3937**

*Dickeya* spp. are Gram-negative  $\gamma$ -proteobacteria, facultative anaerobic bacteria, members of the Enterobacteriaceae family, causing soft-rot diseases in important agricultural crops (Agrios, 2005). *Dickeya* species have been restricted to tropical regions for a long time, but nowadays they have spread across the globe, earning a spot inside the top 10 most important plant pathogens in the world

(Mansfield et al., 2012). Interestingly, bacteria from the *Dickeya* species can also infect insects, which can serve as dissemination vectors (Grenier et al., 2006; Paliwal et al., 2022).

*Dickeya dadantii* 3937 (Dd3937), isolated from African violet (*Saintpaulia ionantha*), is considered a model organism within the *Dickeya* species, mainly due to its easy genetic manipulation. In addition, because apart from infecting a wide variety of crops, including potato, also infects the model plant system *A. thaliana* (Reverchon et al., 2016). It can infect different plant structures, like leaves, tubers, rhizomes, and stems.

Dd3937 can live as an epiphyte or as a saprophyte in soil and ground water until it encounters a susceptible host (Reverchon & Nasser, 2013). Briefly, there are four main steps of plant infection by *D. dadantii*: (1) adherence to plant surface and entry into the plant tissue, either through wounds or natural openings like stomata; (2) colonization of the apoplastic spaces between plant cells; (3) suppression of the host defense response; and (4) plant cell wall degradation resulting in symptom development (Figure 15). This latter step is caused by the activity of cell wall-degrading enzymes (CWDEs), including pectinases (Hugouvieux-Cotte-Pattat et al., 2014), a glucuronoxylanase XynA (Keen, 1996), a periplasmic endogalactanase GanA (Delangle et al., 2007), a cellulase CelZ (Py et al., 1991), and four proteases PrtA, PrtB, PrtC, and PrtG (Wandersman et al., 1987). These CWDEs comprise the main virulence factor of Dd3937 and are secreted by the type I and type II secretion systems. In fact, the pathogenicity mode of *Dickeya* has been termed “brutal force”, as the CWDEs physically attack the cell walls of plant cells and their surrounding apoplast (Toth & Birch, 2005).

To achieve entry into the plant apoplast, Dd3937 requires motility and chemotaxis, which allows location of plant entry sites (Antúnez-Lamas et al., 2009). Once inside the plant, Dd3937 must face and respond to many stress conditions, mainly acidic shock, microaerophilic conditions, low iron availability, and ROS and antimicrobial peptides produced by the plant host (Jiang et al., 2016; Llama-Palacios et al., 2003, 2005; Reverchon et al., 2016; Río-Alvarez et al., 2012). Dd3937 has evolved many strategies to overcome these stress conditions and even make use of them to inflict more damage to the plant. For instance, most genes encoding pectate lyases (Pel) are repressed under acidic conditions, which could mimic the initial stages of infection, while retaining their optimum activity at alkaline conditions (pH 8.9-9.5) (Hugouvieux-Cotte-Pattat et al., 2014). This correlates with the requirement of this kind of enzymes at latter stages of the infection when pH turns slightly basic (Jiang et al., 2016). Contrarily to the other *pel* genes, the *pelA* gene is induced in acidic conditions (Nachin & Barras, 2000), and its pectate lyase activity is very low. However, a *pelA* mutant is significantly less virulent than a wild-type strain. This could appear to be contradictory, but the PelA could have a role in the initial stages of infection initiating tissue degradation weakly (Boccarda et al., 1988). In fact, the downregulation of most genes encoding CWDEs in conditions mimicking the initial stages of infection could be to prevent an early detection of plant degradation products by the host, which would

reveal the presence of the pathogen before it achieves a suitable bacterial population for a successful infection (D'Ovidio et al., 2004).

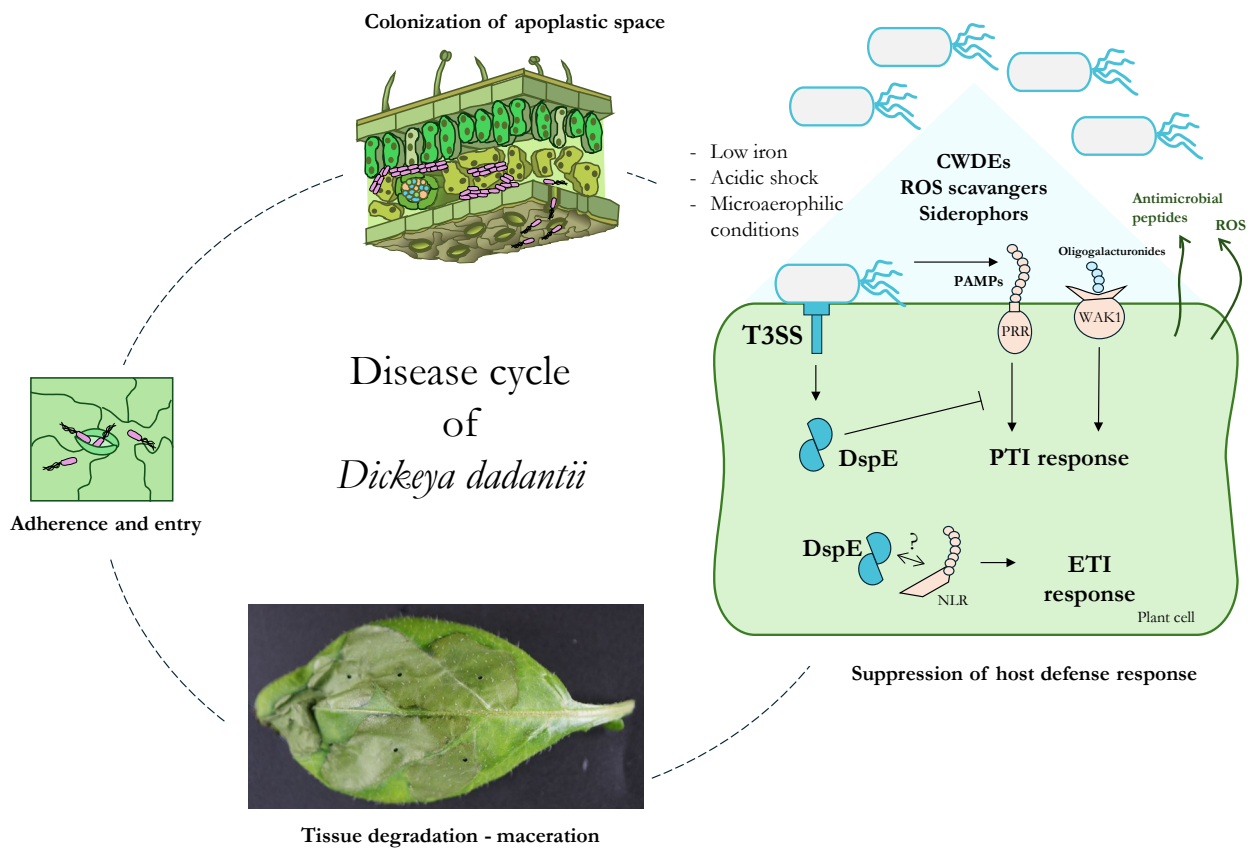


**Figure 15. Disease cycle of *Dickeya dadantii*.** The infection process comprises four different stages: adherence and entry into the plant apoplast, colonization of the apoplastic space, suppression of the host defense response, and tissue degradation. CWDEs, cell-wall degrading enzymes; ETI, effector-triggered immunity; PAMPs, pathogen-associated molecular patterns; PTI, pattern-triggered immunity; ROS, reactive oxygen species; T3SS, type III secretion system. Adapted from Reverchon et al. (2016).

The “brutal force” pathogenicity mode exhibited by Dd3937 is significantly different from other bacterial plant pathogens, like the other pathogen covered in this work *P. syringae*, which uses “stealth” mechanisms to attack plants such as type III-secreted effectors that suppress plant defenses (Xin & He, 2013). Regardless of this difference, bacteria from the *Dickeya* species also encode genes for a T3SS. However, only one canonical effector protein is known for Dd3937, DspE, which belongs to the AvrE superfamily of type III effectors (T3Es). Moreover, it also contains two hairpins, HrpN and HrpW (Choi et al., 2013), also secreted by the T3SS. However, while DspE functions inside the plant cell, these hairpins target the extracellular space of plant tissues. Furthermore, it is thought that the hairpins help translocate the DspE effector inside the plant cells (Glasner et al., 2011). It has also been speculated that hairpins might create pores in the plant cell membranes, promoting cellular death (Oh et al., 2007). HrpN was observed to be important at initial stages of infection (Bauer, 1995; Yang

et al., 2002), and later on it was found to mediate cell aggregation in Dd3937 (Mee-Ngan et al., 2006). HrpW contains a pectate lyase domain and can bind calcium pectate, but it does not present a pectate lyase activity (Choi et al., 2013). Therefore, it has been speculated that HrpW could dock bacteria to pectin, promoting bacterial-plant contact.

Dd3937 experiences low oxygen availability once inside the plant, which is one of the key factors in the development of soft-rot diseases. In fact, pectate lyase synthesis is induced under anaerobic conditions (Hugouvieux-Cotte-Pattat et al., 1992). A transcriptomic analysis revealed that 7% of Dd3937 genes were differentially expressed under anaerobic conditions. These include, apart from anaerobic metabolism related genes, genes involved in responses to environmental stress, metal homeostasis, motility, and energy taxis. This study revealed how at least one putative energy taxis chemoreceptor in Dd3937 was up-regulated and one down-regulated under anaerobic conditions (Babujee et al., 2012).

Dd3937 presents one chemotaxis cluster and 47 chemoreceptors, of which 44 have transmembrane regions, whereas the remaining three are cytoplasmic (Gumerov et al., 2024). The majority of the chemoreceptors present an LBD of the parallel helix type, such as TarH (17 chemoreceptors), 4HB (12), HBM (4), and NIT (1) domains. Some form the curved  $\beta$ -sheet, such as dCache\_1 (2), sCache\_2 (2), and Cache\_3-Cache\_2 (1). Four contain a PAS-domain as LBD, one contains a FIST-type LBD, three present an unknown LBD type, and one lacks a recognizable LBD (Gálvez-Roldán et al., 2023). The transmembrane chemoreceptors belong to the 36H class, as well as the cytoplasmic chemoreceptor without a recognizable LBD. The cytoplasmic chemoreceptor containing two PAS domains belongs to the 24H class, and the one with a FIST-type LBD belongs to the 52H class.

Chemotaxis and motility have been found to be crucial for Dd3937 infection, as mutant strains lacking essential chemotaxis and motility genes had an impaired infection capacity (Antúnez-Lamas et al., 2009). Dd3937 perceives chemical signals derived from the plant to locate entry sites and move in their direction. Several chemoreceptors have been found to be involved in perception of plant derived compounds and have an impact on pathogenesis, like jasmonic acid (Antúnez-Lamas et al., 2009), xylose (Antúnez-Lamas et al., 2009), and nitrate (Gálvez-Roldán et al., 2023). Moreover, the expression of several chemoreceptor genes was found to be altered in response to conditions encountered *in planta* (Jiang et al., 2015). This shows the important role of chemoperception during the plant infection process in Dd3937.

## Objectives



The general objective of this thesis is to deepen our knowledge on bacterial chemoperception in the context of plant pathogenesis, using two model plant pathogens: *Pseudomonas syringae* pv. tomato DC3000 (PsPto) and *Dickeya dadantii* 3937 (Dd3937). As it has been introduced before, these two bacteria are very different in pathogenicity styles, but also in their chemoperception mechanisms. Even though they possess a similar number of chemoreceptors, PsPto contains four chemosensory systems, while Dd3937 contains a single one. Therefore, all signals perceived by the chemoreceptors of Dd3937 are processed by the same chemosensory system. This is not the case for PsPto, in which its chemoreceptors can associate with different chemosensory systems, displaying a more complex signaling system for chemoperception. Knowledge on how bacteria with multiple chemosensory systems coordinate to give output responses is still scarce. Furthermore, not much is known on the specific plant signals PsPto could be chemotactically exploiting to switch from an epiphytic stage to induce virulence in tomato plants. As mentioned in the introduction, besides the chemoperception of extracellular cues, bacteria also perceive their inner metabolic state and respond accordingly in a process called energy taxis. Information about how energy taxis affects bacterial plant pathogenesis is very scarce. Since Dd3937 presents a facultative metabolism and possess a single chemosensory system, which simplifies the signaling process, we decided to use it as a model organism to tackle this issue. Therefore, the specific objectives of this thesis are:

1. Characterization of three chemosensory systems of *Pseudomonas syringae* pv. tomato DC3000 and how kinase and response regulator coordinate to generate output responses.
2. Functional and biochemical characterization of a chemoreceptor from *Pseudomonas syringae* pv. tomato DC3000 putatively involved in the interaction with plants.
3. Characterization of Aer-like chemoreceptors of *Dickeya dadantii* 3937 in the context of energy taxis and plant pathogenesis.



## **Materials and methods**



## 1. Microbiological methods

### 1.1. Bacterial strains, culture media and growth conditions

Bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown in Lysogeny Broth (LB) at 37°C. *Pseudomonas syringae* pv. tomato DC3000 (PsPto) and *Dickeya dadantii* 3937 (Dd3937) were generally grown in King's B medium (King et al., 1954) (KB) at 28°C. For specific purposes, PsPto and Dd3937 strains were also grown in minimal media, these being M9 minimal media supplemented with 10 mM citrate and M63 minimal media (Malke, 1993) supplemented with 30 mM succinate, respectively. When appropriate, the following antibiotics were added to the media at the following concentrations: ampicillin 100  $\mu\text{g}\cdot\text{ml}^{-1}$ , rifampicin 25  $\mu\text{g}\cdot\text{ml}^{-1}$ , tetracycline 10  $\mu\text{g}\cdot\text{ml}^{-1}$ , streptomycin 50  $\mu\text{g}\cdot\text{ml}^{-1}$ , kanamycin 50  $\mu\text{g}\cdot\text{ml}^{-1}$ , gentamycin 5 and 10  $\mu\text{g}\cdot\text{ml}^{-1}$ . For Bacterial Adenylate Cyclase Two Hybrid Assays, which will be described in the following sections, LB media was also supplemented with 0.5 mM isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) 40  $\mu\text{g}\cdot\text{ml}^{-1}$ . Bacterial growth was monitored by measuring the optical density at 600 nm ( $\text{OD}_{600}$ ) using a Jenway 6300 spectrophotometer.

### 1.2. Growth curves

Bacterial growth curves were performed in a 100-well lidded Honeycomb Microplate using a Bioscreen °C Pro. Bacterial strains were first grown in KB agar and then washed twice at  $5000 \times g$  for 5 minutes with KB or minimal media (M9 or M63) supplemented with a carbon source (10 mM citrate or 30 mM succinate) and adjusted to an  $\text{OD}_{600}$  of 0.05 in a 100-well lidded Honeycomb Microplate, which was introduced into Bioscreen °C Pro for growth monitoring at  $\text{OD}_{600}$ .

Growth curves were also performed in Erlenmeyer flasks in a final volume of 50 ml by manually measuring the  $\text{OD}_{600}$  every hour for seven consecutive time-points and one final time-point at 24 hours.

### 1.3. Swimming motility assays

Individual colonies grown on KB agar plates were inoculated with a toothpick into soft agar (0.3% wt·vol<sup>-1</sup>) KB plates, M9 supplemented with 10 mM citrate plates, *hrp*-inducing media (HIM) (Huynh et al., 1989) plates, or M63 supplemented with 30 mM succinate plates. Plates were incubated at 28°C in a humid environment for different timepoints, ranging from 24 to 72 hours. Pictures of the swimming colony were taken, and the diameter of swimming area was measured.

## 1.4. Twitching motility assays

Individual colonies of PsPto strains were inoculated in plates containing 10 ml of KB agar at 1% wt·vol<sup>-1</sup>. Plates were left at room temperature upside down for 24 hours before the experiment. Inoculation was performed by stabbing the agar with a toothpick making sure to reach the bottom of the plate. Plates were incubated at 28°C in a humid environment and under darkness conditions for 5 days.

Surface biomass was carefully removed by washing with distilled water. Plates were then flooded with TM revealing solution (50% methanol, 10% acetic acid, 40% water) and left at room temperature (RT) until twitching halo was observed. TM revealing solution was discarded and diameter of twitching area was measured.

## 1.5. Biofilm attachment assays

Overnight cultures of PsPto grown in KB were washed three times at  $5000 \times g$  for 5 minutes in MGA liquid medium (54 mM mannitol, 3.6 mM KH<sub>2</sub>PO<sub>4</sub>, 23 mM NaCl, 0.8 mM MgSO<sub>4</sub>, 18 mM NH<sub>4</sub>Cl; pH 7.0) supplemented with 2 mM CaCl<sub>2</sub> and adjusted to final OD<sub>600</sub> of 0.02. 100 µl of bacterial suspensions were added to 96-well plates and incubated statically at RT 48 h. Planktonic cells were removed from wells and wells were gently washed 3 times with distilled water. 150 µl of 0.1% (wt·vol<sup>-1</sup>) crystal violet stain was added to each well and incubated for 30 min. After three gentle washes with distilled water, stained biofilms were resuspended in 30% (vol·vol<sup>-1</sup>) acetic acid. The Abs<sub>570</sub> was recorded for each well and the average and standard deviation for technical replicates were computed.

The same procedure was followed for Dd3937 strains with some modifications. The strains were grown overnight in KB plates supplemented with the appropriate antibiotics and washed three times at  $5000 \times g$  for 5 minutes with SOBG medium (20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 0.186 g of KCl, 2.4 g of MgSO<sub>4</sub>·7H<sub>2</sub>O and 50 mL of 40 % glycerol per liter). Static cultures, prepared in the same manner as for PsPto strains, were incubated at 37°C for 48 hours.

## 1.6. Colony morphology and Congo red binding assays

Overnight cultures of PsPto were washed with KB medium and diluted to an OD<sub>600</sub> of 0.05. 20 µl of diluted culture were inoculated either in KB agar plates (1.5% wt·vol<sup>-1</sup>) supplemented with Congo Red (40 µg·ml<sup>-1</sup>) and Coomassie Brilliant Blue (20 µg·ml<sup>-1</sup>) or M9 citrate agar plates (1.5% wt·vol<sup>-1</sup>) supplemented with Congo Red (40 µg·ml<sup>-1</sup>). Pictures were taken with ZEISS Axio Zoom.V16 stereoscope at different time points.

## 1.7. Flocculation assays

PsPto strains were grown overnight in KB medium and left statically at RT for 24 hours. Pictures were taken overnight.

## 1.8. Quantitative capillary chemotaxis assays

PsPto strains were grown overnight in KB medium, diluted to an OD<sub>600</sub> of 0.08 with fresh KB medium in a total volume of 50 ml, and left to grow at 28°C until it reached an OD<sub>600</sub> of 0.25. While bacterial cultures grew, chemoattractant solutions were prepared in 10 mM HEPES. Cultures were washed twice with 10 mM HEPES buffer for 5 minutes at  $1740 \times g$  and adjusted to an OD<sub>600</sub> of 0.25. 230  $\mu$ l of bacterial suspension were distributed into the appropriate wells of a 96-well plate. 1- $\mu$ l capillaries were first heated gently and filled with the appropriate chemoattractant, or buffer as a negative control, placed into the well containing the bacterial suspension and incubated for 30 minutes. The capillary was washed with milliQ water and introduced into 1 ml of KB medium, breaking the capillary if possible. Eppendorf tubes with the capillary inside were spined down to release the bacteria that entered the capillary into the medium. Four serial dilutions were performed, and 80  $\mu$ l were drop plated onto KB plates supplemented with the appropriate antibiotics distributed in 4 drops of 20  $\mu$ l each. Plates were incubated at 28°C until colonies appeared, and counted to determine how many bacteria entered the capillary in contact with the chemoattractant.

Dd3937 strains were grown in KB agar plates, scraped out, resuspended with MA buffer (7 g of K<sub>2</sub>HPO<sub>4</sub>, 3 g of KH<sub>2</sub>PO<sub>4</sub>, 0.1 g of Mg<sub>2</sub>SO<sub>4</sub>·7H<sub>2</sub>O, and 1 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> per liter; pH 7) and washed twice with MA buffer for 5 minutes at  $1740 \times g$ . The chemoattractant solutions were prepared in MA buffer. The rest of the protocol is the same as for PsPto strains.

## 1.9. Air trap assays

Aerotaxis was assayed based on the air trap scheme of Arrebola & Cazorla (2020) and Tumewu et al. (2022), with some modifications. UV sterilized Pasteur pipettes were placed in sterilized glass tubes. 9.5 mL of M63 30 mM succinate 0.1% agar were added to the tubes outside of the Pasteur pipette. Dd3937 strains were grown overnight at 28°C in M63 supplemented with 30 mM succinate, washed once with fresh M63 supplemented with 30 mM succinate for 3 minutes at  $1740 \times g$ , and adjusted to an OD<sub>600</sub> of 0.5. 500  $\mu$ l of the adjusted bacterial suspension were added to a final volume of 10 ml of media outside of the Pasteur pipette. After inoculation, 2 ml of paraffin oil were added to the tube with special care of avoiding the inside of the Pasteur pipette. In this way, air exchange was avoided in the broth but not in the Pasteur pipette, thus forming an air trap. Air trap tubes were statically incubated at 28°C for 24 to 48 hours. Pictures of the air-liquid interphase were taken, and 150  $\mu$ l of the bacterial

suspension from inside the Pasteur pipette were collected. Bacterial populations were determined by serial dilution and drop plating onto KB plates with the appropriate antibiotics.

## **2. Molecular Biology and Biochemistry methods**

### **2.1. Cloning methods**

#### **2.1.1. Construction of LBD expression clones**

The LBD domain of the PsPto-PscC (PSPTO\_2448) was defined as the protein fragment in between two transmembrane regions, determined by the TMHMM server v. 2.0 (Krogh et al., 2001) and the DAS transmembrane region prediction algorithm (Cserzo et al., 1997).

A pET28(b)+ derivative containing a version of the LBD of *PsPto-PscC* (792 bp) codon optimized for *E. coli* was purchased from Biocat, named p2448-LBD. This plasmid was transformed into *E. coli* BL21 (AI) (Invitrogen) for protein expression and purification assays.

#### **2.1.2. Construction of mutant strains of PsPto**

Mutagenesis in PsPto was performed by truncation or deletion of whole genes or internal regions of a given gene. For truncation, an internal region of the target gene was amplified and cloned into the pKOsac101 vector (Cerna-Vargas et al., 2019). The resulting plasmid was introduced in PsPto by electroporation and mutation was checked by PCR. For gene deletion, approximately 1-kb fragments corresponding to the upstream and downstream regions of the target gene were PCR amplified and fused together by an overlap extension PCR procedure. The resulting ~2-kb fragment was cloned into the small, mobilizable, sucrose-counter selection vector pK18mobsac (Kvitko & Collmer, 2011). The resulting plasmid was checked by PCR and sequencing.

pK18mobsac:: $\Delta$ *target\_gene* was introduced into PsPto by electroporation and first recombination events were selected on KB plates supplemented with kanamycin. Kanamycin resistant colonies were picked and resuspended into 1 ml of KB medium. 50 to 200  $\mu$ l of the resulting bacterial suspension were plated on KB plates supplemented with 10% sucrose to select for second recombination events. Colonies that survived sucrose selection were tested for kanamycin sensitivity, and resulting colonies were checked by PCR.

#### **2.1.3. Construction of mutant strains of Dd3937**

Mutagenesis in Dd3937 was performed by an allelic exchange procedure interrupting the target gene with a transposon which harbors an antibiotic resistance cassette flanked by two transcriptional

terminators. Approximately 1-kb fragments corresponding to the upstream and downstream region of the target gene were PCR amplified and fused together by an overlap extension PCR procedure. An EcoRI site was introduced in between the two fragments. This resulting ~2-kb fragment was cloned into pGEMTeasy vector. The resulting plasmid was checked by PCR and sequencing.

pGEMT::*target\_gene\_Ω\_cassette* was introduced into Dd3937 by electroporation. Transformants were selected in KB plates supplemented with ampicillin and the corresponding antibiotic depending on the transposon used (gentamycin, spectinomycin, or kanamycin). Since pGEMT is replicative in Dd3937, transformants were inoculated in a A-P media (12.14 g of Tris, 0.1 g of Mg<sub>2</sub>SO<sub>4</sub>·7H<sub>2</sub>O, 1 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 0.5 g of sodium citrate per liter; pH 7.4) supplemented with 0.02% (vol·vol<sup>-1</sup>) glycerol and 250 μM potassium phosphate (pH 7) and left to grow for 32 hours. This low-phosphate minimal media forces a plasmid curation process. 10 μl were used to re-inoculate A-P media supplemented with glycerol and potassium phosphate and left to incubate for 32 hours. 100 μl were plated on KB plates supplemented with the appropriate antibiotic depending on the transposon used, and on KB plates supplemented with both the previous antibiotic and ampicillin. This process was repeated until a clear difference could be observed between both selection conditions, where most cells could not survive the presence of ampicillin in the media. Then, colonies were checked for the loss of ampicillin resistance and retainment of transposon resistance. The resulting colonies were finally checked by PCR.

#### 2.1.4. Construction of complemented strains

Mutant of both PsPto and Dd3937 were complemented with pBBR1MCS2 plasmids. Target genes including, when possible, their promoter region, were PCR amplified and cloned into pBBR1MCS2 plasmids. Complementation plasmids were checked by PCR and sequencing. These plasmids were introduced in PsPto or Dd3937 by electroporation and selected with the appropriate antibiotics.

In specific occasions, PsPto strains were complemented using the pME6010 plasmid, following the same cloning strategy.

#### 2.1.5. Construction of Bacterial Adenylate Cyclase Two Hybrid clones

Strains and plasmids for Bacterial Adenylate Cyclase Two Hybrid (BACTH) assays were purchased from Euromedex (France). These plasmids are pKT25, pKNT25, pUT18, and pUT18C, which contain the T25 or the T18 fragment of the adenylate cyclase from *Bordetella pertussis*. Target genes were PCR amplified and cloned in frame with the T25 or the T18 fragments. These plasmids allow for cloning of the target gene at N-terminal or C-terminal of the adenylate cyclase fragments, enhancing the probability of a stable fusion protein. Primers used for this are listed in Table 2.

## 2.2. Bacterial Adenylate Cyclase Two Hybrid assays

The reporter strain *E. coli* DHM10 (*cya*<sup>-</sup>) was co-transformed with two-hybrid plasmids. For negative controls, pKT25-CheA1, pKT25-CheA2, pKT25-CheA3, pKT25-FlhM, and pUT18 were used. For positive controls, pKT25-zip and pUT18-zip were used. Clones were selected on LB agar plates supplemented with ampicillin and kanamycin. Three clones of each co-transformation were inoculated in LB medium supplemented with kanamycin and ampicillin and grown at 37°C under shaking conditions for at least 4 hours. 2 µl of culture were spotted either on LB plates supplemented with kanamycin, ampicillin, IPTG, and X-gal. Plates were incubated for 3 days at 28°C.

## 2.3. RNA extraction from bacterial cultures

PsPto strains were grown at 28°C in HIM with shaking at 150 rpm to an OD<sub>600</sub> of 0.5. When necessary, GABA at a final concentration of 1 mM was added and cultures were incubated at 28°C for 2 h. Cells were then collected by centrifugation at 5000 × g for 5 min, the supernatant was discarded, and the pellets were stored at -80°C. Total RNA was extracted using TRI reagent solution (ThermoFisher Scientific) following the manufacturer's instructions. The purification was accomplished using the High Pure RNA Isolation Kit (Roche) following the manufacturer's recommendations. RNA samples were quantified using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Inc.).

## 2.4. Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was isolated as described above and converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers were designed to amplify fragments of approximately 100 bp (Table 2) and the *rpoD* gene was used as an internal control (Sawada et al., 1999). Reverse transcription-quantitative PCR (RT-qPCR) amplifications were carried out on an ABI PRISM 7300 RT PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). Thermal cycling conditions were as follows: one cycle at 95°C for 10 min; 50 cycles at 95°C for 15 s and 60°C for 1 min; and a final cycle at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s. The relative gene expression ratio was calculated using the comparative critical threshold ( $\Delta\Delta C_t$ ) method (Pfaffl, 2001; Rotenberg et al., 2006).

## 2.5. Protein expression and purification

*E. coli* BL21 AI were transformed with p2448-LBD and selected on LB plates supplemented with kanamycin. Transformant colonies were picked and inoculated in liquid LB media supplemented with kanamycin and left to grow at 37°C until cultures reached an OD<sub>600</sub> of 1. The cultures were then

diluted to an OD<sub>600</sub> of 0.05 and left to grow at 28°C until they reached an OD<sub>600</sub> of 0.5-0.6. Protein expression was induced with 0.5 mM IPTG and cultures were left to grow at 16°C. Cells were harvested by centrifugation at 10,000 × *g* for 30 minutes at 4°C. Cell pellets were resuspended in buffer A (30 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, 10% [vol·vol<sup>-1</sup>] glycerol, pH 8.0), disrupted by sonication, and centrifuged at 13,000 × *g* for 1 hour. The supernatant was then loaded onto a 5-ml HisTrap column (Amersham Biosciences) previously equilibrated with 5 column volumes of buffer A, which was subsequently washed with buffer A containing 10 mM imidazole and eluted with a linear gradient of 10 to 500 mM imidazole in buffer A. Protein-containing fractions were pooled and dialyzed into buffer B (50 mM HEPES, 300 mM NaCl, 10% [vol·vol<sup>-1</sup>] glycerol, pH 8.0) for immediate analysis.

## 2.6. Thermal shift assays

Thermal shift assays were performed on a Bio-Rad MyIQ2 real-time PCR instrument. Ligands from the compound array (Biolog, Hayward, CA, USA) were dissolved in 50 µL of Milli-Q water, which, according to the manufacturer, corresponds to a concentration of 10 to 20 mM.

Screening was performed using 96-well plates. Assay mixtures (25 µL) contained dialyzed protein, SYPRO orange (Life Technologies) at a 5× concentration, and ligands at final concentrations of 1 to 2 mM. In a single well (ligand-free protein), the compound was replaced by water. Samples were heated from 23°C to 85°C at a scan rate of 1°C per minute. The protein unfolding curves were obtained by monitoring the changes in SYPRO orange fluorescence. Melting temperatures were determined using the first derivative values from the raw fluorescence data.

## 2.7. Isothermal Titration Calorimetry

These experiments were conducted by collaborators in the Estacion Experimental del Zaidín (Granada, Spain). Experiments were conducted on a VP-ITC microcalorimeter (MicroCal) at 25°C. PsPto-PscC-LBD was dialyzed overnight against buffer B, adjusted to a concentration of 13 µM, and placed into the sample cell of the instrument. The protein was titrated by the injection of 4.2–8.6-µl aliquots of 500 µM ligand solutions that were prepared in buffer B (50 mM HEPES, 300 mM NaCl, 10% [vol·vol<sup>-1</sup>] glycerol, pH 8.0) immediately before use. The mean enthalpies measured from the injection of ligands into buffer were subtracted from raw titration data prior to data analysis using the “one-binding-site” model of the MicroCal version of ORIGIN.

### 3. Phytopathology methods

#### 3.1. Entry assays in tomato plants

PsPto strains were grown at 28°C for 48 h on KB agar in darkness. Cells were resuspended, washed twice with 10 mM MgCl<sub>2</sub> at 5000 × *g* for 5 minutes and diluted to obtain a bacterial suspension containing 10<sup>8</sup> cfu·ml<sup>-1</sup>. Three-week-old tomato plants (*Solanum lycopersicum* cv. Moneymaker) were sprayed with the suspension after addition of Silwet L-77 at a final concentration of 0.02% (vol·vol<sup>-1</sup>).

Plants were incubated in a growth chamber at 25°C and 60% relative humidity (RH) with a daily light period of 12 hours. Bacterial populations were determined at 2 h postinoculation by sampling five 1-cm-diameter leaf disks per plant. The infected leaf disks were washed twice with 10 mM MgCl<sub>2</sub> prior to homogenization to eliminate the bacteria from the leaf surface. Plant material was homogenized in 10 mM MgCl<sub>2</sub> and drop plated onto KB agar supplemented with the appropriate antibiotics. The average number of bacteria per square centimeter isolated from five infected tomato leaves was determined based on log-transformed data. A surface sterilization procedure (Romero et al., 2019) was carried out prior to plant material homogenization. Briefly, leaves were surface-sterilized with a 5% (vol·vol<sup>-1</sup>) solution of commercial bleach and 0.01% (vol·vol<sup>-1</sup>) of Tween 20 for 10 min and finally rinsed three times with sterile distilled water. The final wash was plated on KB plates to confirm the absence of bacteria.

#### 3.2. Virulence assays in tomato plants

PsPto strains were grown at 28°C for 48 h on KB agar in darkness. Cells were resuspended in 10 mM MgCl<sub>2</sub> and diluted to 10<sup>8</sup> cfu·ml<sup>-1</sup>. Three-week-old tomato plants (*Solanum lycopersicum* cv. Moneymaker) were sprayed with the bacterial suspension after addition of Silwet L-77 at a final concentration of 0.02% (vol·vol<sup>-1</sup>).

Plants were incubated in a growth chamber at 25°C and 60% RH with a daily light period of 12 hours. Six days after inoculation, the leaf symptoms were recorded, and bacterial populations from three plants were measured.

#### 3.3. Virulence assays in *Arabidopsis thaliana*

PsPto strains were grown at 28°C for 48 hours in KB agar in darkness. Cells were resuspended in 10 mM MgCl<sub>2</sub>, washed twice at 5000 × *g* and diluted to 3×10<sup>8</sup> cfu·ml<sup>-1</sup>. Four-week-old *A. thaliana* Col-0 or *gad1/2* double mutant (Scholz et al., 2015a) plants were spray-inoculated with the bacterial suspension. Six days after inoculation, the leaf symptoms were recorded, and bacterial

populations from three plants were measured. Plant material was processed as described for tomato plants.

### **3.4. Entry assays in potato plants**

Dd3937 strains were grown on KB agar plates at 28° for 24 hours. Cells were washed twice with 10 mM MgCl<sub>2</sub> at 5000 × g for 5 minutes and diluted to 5×10<sup>7</sup> cfu·ml<sup>-1</sup>. Three-week-old *Solanum tuberosum* ('Desiree') leaves were pierced 6 times with a needle on the adaxial side of leaves. Inoculation of the leaves was performed by placing a 10 µl-drop of bacterial suspension on top of each hole. 5 leaves were inoculated for each plant and plants were incubated in a growth chamber at 28°C and 80% RH with a daily light period (100 µE·[m<sup>2</sup> s]<sup>-1</sup>) of 12 h. 24 h postinoculation (hpi), leaf symptoms were recorded, and bacterial population was determined by sampling five 1-cm leaf disks per plant. Leaf disks were washed twice with 10 mM MgCl<sub>2</sub> to eliminate bacteria on the surface and subsequently homogenized in 1 ml of 10 mM MgCl<sub>2</sub>. Serial dilutions were performed and drop-plated on KB agar plates supplemented with the appropriate antibiotics.

### **3.5. Virulence assays in potato plants**

The same protocol as in the entry assays was followed, only modifying the inoculation method. In this case, plant leaves were inoculated by syringe infiltration. 5 leaves per plant were syringe-inoculated with 100 µl of bacterial suspension diluted to 5×10<sup>7</sup> cfu·ml<sup>-1</sup>. Bacterial populations were recorded following the same protocol as in the entry assays. Symptoms were recorded and the macerated area was measured using the software GIMP. The number of infections that progressed and generated symptoms were also recorded.

## **4. Cryo-electron microscopy methods**

### **4.1. Sample preparation**

PsPto strains were grown overnight in KB liquid media supplemented with Rf25 at 28°C. Cells were washed twice at 3000 × g for 5 minutes with M9 minimal media supplemented with 10 mM citrate and concentrated by centrifugation at 3,000 × g for 5 minutes. Protein A-treated 10-nm colloidal gold solution (Cell Microscopy Core, Utrecht University, Utrecht, The Netherlands) were added to the samples diluted 20 times and mixed gently before plunge-freezing. Subsequently, 3 µl of the sample was applied to a freshly glow-discharged, carbon-coated copper grid R2/2, 200 mesh grid (Quantifoil Micro Tools GmbH, Großlobichau, Germany). Plunge-freezing was performed on an EM GP plunge

freezer (Leica Microsystems, Wetzlar, Germany) in liquid ethane under the following conditions: 30 seconds of pre-blotting time, 1 second of blotting time, 85% of humidity, 20°C of chamber temperature.

## 4.2. Cryo-electron tomography

Data was acquired using a TITAN Krios 300 kV cryo-transmission electron microscope (Thermo Fisher Scientific, Hillsboro, OR, USA) and images recorded with a K3 direct electron detector (Bioquantum, Gatan, Pleasanton, CA) with a slit width of 20 eV. Images were acquired at a magnification of  $\times 19,500$ , which corresponds to a pixel size of 4,411 Å. Tilt series were collected using SerialEM 4.1 (Mastronarde, 2005) with a bidirectional tilt scheme from  $-60^\circ$  to  $60^\circ$ , starting from  $0^\circ$  with  $2^\circ$  increments. The cumulative exposure was set to  $157 \text{ e}^- \cdot \text{Å}^{-1}$  and the defocus to  $-6 \text{ }\mu\text{m}$ . From each strain 48 – 50 tilt series were collected and used to perform three-dimensional reconstruction using the IMOD software package (Kremer et al., 1996; Mastronarde, 2008). The tomograms were screened for the presence of visible chemotaxis arrays. Only tomograms with chemotaxis arrays in sideview were further processed.

## 4.3. Measurement of chemotaxis arrays from cryo-tomograms

The measurement of the average array density profiles was calculated automatically using the "sideview-profile-average" script (Ortega et al., 2020). Briefly, model points were generated in tomograms along the inner membrane and chemotaxis arrays. Based on these model points, the script calculates the average pixel value perpendicular to the model points in the same plane to create *json* files with the average pixel profile. The profiles were visualized in ObservableHQ notebook to measure the distance between the density dips of the inner membrane and the CheA/CheW plane.

# 5. Statistical analysis

Statistical analyses were performed using the GraphPad Prism software. First, distribution of the population was determined using Kolmogorov-Smirnov normality tests. When samples followed a normal distribution, a t-test or one-way analysis of variance (ANOVA) were used to determine significant differences among averages, depending on the sample group. When ANOVA was used, multiple comparisons were performed using Fisher's least significant difference (LSD) post hoc test. When samples did not follow a normal distribution, a Man-Whitney test or Kruskal-Wallis were used to determine significant differences among averages, depending on the sample group. When non-parametric group comparisons were used, multiple comparisons were performed using Dunn's test.

Table 1. Bacterial strains and plasmids used in this study.

| Strains and plasmids                                 | Relevant characteristics <sup>a</sup>                                                                                                                                                                 | Reference or source    |
|------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------|
| <b><i>Escherichia coli</i></b>                       |                                                                                                                                                                                                       |                        |
| DH5 $\alpha$                                         | <i>supE44 lacU169 (Ø80lacZΔ M15) hsdR17 (r<sub>k</sub>-m<sub>k</sub>-) recA1 endA1 gyrA96 thi-1 relA1</i>                                                                                             | (Hanahan, 1983)        |
| CC118λpir                                            | <i>Sp<sup>r</sup>, Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE recA1 lysogenized with λpir phage</i>                                                                                | (Herrero et al., 1990) |
| BL21 (AI)                                            | <i>F<sup>-</sup> ompT gal dcm lon hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>) λ(DE3 [lacI lacUV5 T7p07 ind1 sam7 nin5]) [malB<sup>+</sup>]<sub>K-12</sub>(λ<sup>S</sup>)</i> | Invitrogen, CA, USA    |
| DHM1                                                 | <i>F<sup>-</sup> cya-854 recA1 endA1 gyrA96 (Nal<sup>r</sup>) thi1 hsdR17 spoT1 rfbD1 glnV44(AS)</i>                                                                                                  | Euromedex, France      |
| <b><i>Pseudomonas syringae</i> pv. tomato DC3000</b> |                                                                                                                                                                                                       |                        |
| PsPto                                                | Wild-type; R <sup>f</sup>                                                                                                                                                                             | (Cuppels, 1986b)       |
| Δ <i>cheA1</i>                                       | Deletion of the first 1032 bp of <i>PSPTO_0913</i> and 21 bp from its intergenic region                                                                                                               | This study             |
| Δ <i>cheA2</i>                                       | Deletion of the first 1214 bp from <i>PSPTO_1982</i>                                                                                                                                                  | This study             |
| Δ <i>cheA3</i>                                       | In-frame deletion of 2343 bp from <i>PSPTO_1497</i>                                                                                                                                                   | This study             |
| Δ <i>cheY1</i>                                       | In-frame deletion of 348 bp from <i>PSPTO_0910</i>                                                                                                                                                    | This study             |
| Δ <i>cheY2</i>                                       | In-frame deletion of 336 bp from <i>PSPTO_1980</i>                                                                                                                                                    | This study             |
| Δ <i>wspR</i>                                        | Complete deletion of <i>PSPTO_1980</i> gene and 18 bp of its intergenic region                                                                                                                        | This study             |
| Δ <i>cheW-F6</i>                                     | In-frame deletion of 1413 bp from <i>PSPTO_1987</i> and <i>PSPTO_1988</i> , including their intergenic region                                                                                         | This study             |
| Δ <i>0912</i>                                        | Deletion of the complete sequence of <i>PSPTO_0912</i> (1656 bp)                                                                                                                                      | This study             |
| PsPto-pBBR1MCS-2                                     | PsPto harboring the pBBR1MCS-2 plasmid                                                                                                                                                                | This study             |
| PsPto-pME6010                                        | PsPto harboring the pME6010 plasmid                                                                                                                                                                   | This study             |
| Δ <i>cheA1</i> -C                                    | Δ <i>cheA1</i> harboring wild type <i>cheA1</i> in plasmid; pME6010::CheA1; Tet <sup>r</sup>                                                                                                          | This study             |
| Δ <i>cheA2</i> -C                                    | Δ <i>cheA2</i> harboring wild type <i>cheA2</i> in plasmid; pME6010::CheA2; Tet <sup>r</sup>                                                                                                          | This study             |
| Δ <i>cheA3</i> -C                                    | Δ <i>cheA3</i> harboring wild type <i>cheA3</i> in plasmid; pME6010::CheA3; Tet <sup>r</sup>                                                                                                          | This study             |
| Δ <i>cheY2</i> -C                                    | Δ <i>cheY2</i> harboring wild type <i>cheY2</i> in plasmid; pBBR1MCS2::CheY2; Km <sup>r</sup>                                                                                                         | This study             |
| Δ <i>wspR</i> -C                                     | Δ <i>wspR</i> harboring wild type <i>wspR</i> in plasmid; pME6010::WspR; Tet <sup>r</sup>                                                                                                             | This study             |

|                                     |                                                                                                                                                                     |                                                |
|-------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------|
| PsPto- <i>pscC</i>                  | <i>PSPTO_2448</i> ::pKOSac10; Sm <sup>r</sup>                                                                                                                       | This study, (Santamaría-Hernando et al., 2022) |
| PsPto- <i>pscC</i> -C               | PsPto- <i>pscC</i> complemented with pBBR1MCS-2::PSPTO_2448; Sm <sup>r</sup> , Km <sup>r</sup>                                                                      | This study, (Santamaría-Hernando et al., 2022) |
| <b><i>Dickeya dadantii</i> 3937</b> |                                                                                                                                                                     |                                                |
| Dd3937                              | Wild-type                                                                                                                                                           | (Kotoujansky et al., 1985)                     |
| <i>17070</i>                        | <i>17070</i> ::ΩKm cassette; Km <sup>r</sup>                                                                                                                        | This study                                     |
| <i>12290</i>                        | <i>12290</i> ::ΩKm cassette; Km <sup>r</sup>                                                                                                                        | This study                                     |
| <i>12165</i>                        | <i>12165</i> ::ΩGm cassette; Gm <sup>r</sup>                                                                                                                        | This study                                     |
| <i>17070-12290</i>                  | <i>17070</i> ::ΩKm cassette, <i>12290</i> ::ΩSp cassette; Km <sup>r</sup> , Sp <sup>r</sup>                                                                         | This study                                     |
| <i>17070-12165</i>                  | <i>17070</i> ::ΩKm cassette, <i>12165</i> ::ΩGm cassette; Km <sup>r</sup> , Gm <sup>r</sup>                                                                         | This study                                     |
| <i>12165-12290</i>                  | <i>12165</i> ::ΩGm cassette, <i>12290</i> ::ΩKm cassette; Gm <sup>r</sup> , Km <sup>r</sup>                                                                         | This study                                     |
| <i>17070-12290-12165</i>            | <i>17070</i> ::ΩKm cassette, <i>12290</i> ::ΩSp cassette, <i>12165</i> ::ΩGm cassette; Km <sup>r</sup> , Sp <sup>r</sup> , Gm <sup>r</sup>                          | This study                                     |
| <b>Plasmids and clones</b>          |                                                                                                                                                                     |                                                |
| pK18mobsac                          | oriColE1, <i>sacB</i> ; Km <sup>r</sup>                                                                                                                             | (Kvitko & Collmer, 2011)                       |
| pK18mobsac:: <i>cheA1</i> -KO       | pK18mobsac plasmid harboring 1006 bp upstream of <i>PSPTO_0913</i> fused to 1023 bp of an internal region of <i>PSPTO_0913</i> cloned at XmaI site; Km <sup>r</sup> | This study                                     |
| pK18mobsac:: <i>cheA2</i> -KO       | pK18mobsac plasmid harboring 1070 bp upstream of <i>PSPTO_1982</i> fused to 1006 bp of <i>PSPTO_1982</i> cloned at XmaI site; Km <sup>r</sup>                       | This study                                     |
| pK18mobsac:: <i>cheA3</i> -KO       | pK18mobsac plasmid harboring 996 bp upstream of <i>PSPTO_1497</i> fused to 1011 bp downstream of <i>PSPTO_1497</i> cloned at XmaI site; Km <sup>r</sup>             | This study                                     |
| pK18mobsac:: <i>cheY1</i> -KO       | pK18mobsac plasmid harboring 963 bp upstream of <i>PSPTO_0910</i> fused to 953 bp downstream of <i>PSPTO_0910</i> cloned at XmaI site; Km <sup>r</sup>              | This study                                     |
| pK18mobsac:: <i>cheY2</i> -KO       | pK18mobsac plasmid harboring 977 bp upstream of <i>PSPTO_1980</i> fused to 949 bp downstream of <i>PSPTO_1980</i> cloned at XmaI site; Km <sup>r</sup>              | This study                                     |
| pK18mobsac:: <i>wspR</i> -KO        | pK18mobsac plasmid harboring 1041 bp upstream of <i>PSPTO_1499</i> fused to 1041 bp downstream of <i>PSPTO_1499</i> cloned at XmaI site; Km <sup>r</sup>            | This study                                     |
| pK18mobsac:: <i>cheW-F6</i> -KO     | pK18mobsac plasmid harboring 1012 bp upstream of <i>PSPTO_1987</i> fused to 1019 bp downstream of <i>PSPTO_1988</i> cloned at XmaI site; Km <sup>r</sup>            | This study                                     |

|                     |                                                                                                                                                                                                                                                  |                       |
|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| pK18mobsac::0912-KO | pK18mobsac plasmid harboring 1013 bp upstream of <i>PSPTO_0912</i> fused to 1024 bp of <i>PSPTO_0912</i> cloned at XmaI site; Km <sup>r</sup>                                                                                                    | This study            |
| pBBR1MCS-2          | <i>oriRK2 mobRK2</i> ; Km <sup>r</sup>                                                                                                                                                                                                           | (Kovach et al., 1995) |
| pBBR1MCS-2::CheY2   | pBBR1MCS-2 harboring a 615-bp PCR fragment containing the <i>PSPTO_1980</i> gene and its promoter region; Km <sup>r</sup>                                                                                                                        | This study            |
| pME6010::CheA1      | pME6010 harboring the complete sequence of <i>PSPTO_0913</i> and 25 bp upstream of its start codon; Tet <sup>r</sup>                                                                                                                             | (Clarke et al., 2016) |
| pME6010::CheA2      | pME6010 harboring the complete sequence of <i>PSPTO_1982</i> and 14 bp upstream of its start codon; Tet <sup>r</sup>                                                                                                                             | (Clarke et al., 2016) |
| pME6010::CheA3      | pME6010 harboring the complete sequence of <i>PSPTO_1497</i> and 40 bp upstream of its start codon; Tet <sup>r</sup>                                                                                                                             | This study            |
| pME6010::WspR       | pME6010 harboring the complete sequence of <i>PSPTO_1499</i> gene and 82 bp upstream of its start codon; Tet <sup>r</sup>                                                                                                                        | This study            |
| pKT25               | plasmid with Plac encoding the T25 fragment of <i>B. pertussis</i> <i>cyaA</i> and a downstream MCS; Km <sup>r</sup>                                                                                                                             | Euromedex, France     |
| pKT25-Zip           | Leucine zipper of GCN4 (4) genetically fused in frame to the T25 fragment in the KpnI site of pKT25; Km <sup>r</sup>                                                                                                                             | Euromedex, France     |
| pKT25-CheA1         | <i>PSPTO_0913</i> was amplified from PsPto DNA and cloned into the PstI site of pKT25 and checked for correct orientation to express T25-CheA1. A “g” was introduced upstream of the start codon to ensure “in-frame” cloning; Km <sup>r</sup>   | This study            |
| pKT25-CheA2         | <i>PSPTO_1982</i> was synthesized de novo and cloned into the XbaI and BamHI sites of pKT25 to express T25-CheA2. A “g” was introduced upstream of the start codon to ensure “in-frame” cloning; Km <sup>r</sup>                                 | Biocat, Germany       |
| pKT25-CheA3         | <i>PSPTO_1497</i> was amplified from PsPto DNA and cloned into the BamHI sites of pKT25 and checked for correct orientation to express T25-CheA3. A “g” was introduced upstream of the start codon to ensure “in-frame” cloning; Km <sup>r</sup> | This study            |
| pKT25-FlIM          | <i>PSPTO_1969</i> was amplified from PsPto DNA and cloned into the BamHI sites of pKT25 and checked for correct orientation to express T25-FlIM. A “g” was introduced upstream of the start codon to ensure “in-frame” cloning; Km <sup>r</sup>  | This study            |
| pKNT25              | Km <sup>R</sup> plasmid with Plac containing a MCS upstream of the T25 fragment of <i>B. pertussis</i> <i>cyaA</i> ; Km <sup>r</sup>                                                                                                             | Euromedex, France     |
| pKNT25-CheA1        | <i>PSPTO_0913</i> was synthesized de novo excluding the stop codon and cloned into the XbaI and BamHI sites of pKNT25 to express CheA1-T25; Km <sup>r</sup>                                                                                      | Biocat, Germany       |
| pKNT25-CheA2        | <i>PSPTO_1982</i> was synthesized de novo excluding the stop codon and cloned into the XbaI and BamHI sites of pKNT25 to express CheA2-T25; Km <sup>r</sup>                                                                                      | Biocat, Germany       |
| pKNT25-CheA3        | <i>PSPTO_1497</i> was amplified from PsPto DNA excluding the stop codon and cloned into the BamHI sites of pKNT25 and checked for correct orientation to express CheA3-T25; Km <sup>r</sup>                                                      | This study            |

|              |                                                                                                                                                                                                                                                  |                   |
|--------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|
| pKNT25-FlIM  | <i>PSPTO_1969</i> was amplified from PsPto DNA excluding the stop codon and cloned into the BamHI sites of pKNT25 and checked for correct orientation to express FlIM-T25; Km <sup>r</sup>                                                       | This study        |
| pUT18        | Amp <sup>R</sup> plasmid with Plac containing a MCS upstream of the T18 fragment of <i>B. pertussis cyaA</i> ; Ap <sup>r</sup>                                                                                                                   | Euromedex, France |
| pUT18-CheY1  | <i>PSPTO_0910</i> was amplified from PsPto DNA excluding the stop codon and cloned into the BamHI sites of pUT18 and checked for correct orientation to express CheY1-T18; Ap <sup>r</sup>                                                       | This study        |
| pUT18-CheY2  | <i>PSPTO_1980</i> was amplified from PsPto DNA excluding the stop codon and cloned into the BamHI sites of pUT18 and checked for correct orientation to express CheY2-T18; Ap <sup>r</sup>                                                       | This study        |
| pUT18-WspR   | <i>PSPTO_1499</i> was amplified from PsPto DNA excluding the stop codon and cloned into the BamHI sites of pUT18 and checked for correct orientation to express WspR-T18; Ap <sup>r</sup>                                                        | This study        |
| pUT18-0912   | <i>PSPTO_0912</i> was amplified from PsPto DNA excluding the stop codon and cloned into the XmaI sites of pUT18 and checked for correct orientation to express 0912-T18; Ap <sup>r</sup>                                                         | This study        |
| pUT18-1493   | <i>PSPTO_1493</i> was amplified from PsPto DNA excluding the stop codon and cloned into the XmaI sites of pUT18 and checked for correct orientation to express 1493-T18; Ap <sup>r</sup>                                                         | This study        |
| pUT18-2014   | <i>PSPTO_2014</i> was amplified from PsPto DNA excluding the stop codon and cloned into the XmaI sites of pUT18 and checked for correct orientation to express 2014-T18; Ap <sup>r</sup>                                                         | This study        |
| pUT18-2441   | <i>PSPTO_2441</i> was amplified from PsPto DNA excluding the stop codon and cloned into the XmaI sites of pUT18 and checked for correct orientation to express 2441-T18; Ap <sup>r</sup>                                                         | This study        |
| pUT18-2480   | <i>PSPTO_2480</i> was amplified from PsPto DNA excluding the stop codon and cloned into the XmaI sites of pUT18 and checked for correct orientation to express 2480-T18; Ap <sup>r</sup>                                                         | This study        |
| pUT18C       | Amp <sup>R</sup> plasmid with Plac encoding the T18 fragment of <i>B. pertussis cyaA</i> and a downstream MCS; Ap <sup>r</sup>                                                                                                                   | Euromedex, France |
| pUT18C-zip   | Leucine zipper of GCN4 (1) genetically fused in frame to the T18 fragment in the KpnI and EcoRI sites of pUT18; Ap <sup>r</sup>                                                                                                                  | Euromedex, France |
| pUT18C-CheY1 | <i>PSPTO_0910</i> was amplified from PsPto DNA and cloned into the BamHI sites of pUT18C and checked for correct orientation to express T18-CheY1. “gg” was introduced upstream of the start codon to ensure “in-frame” cloning; Ap <sup>r</sup> | This study        |
| pUT18C-CheY2 | <i>PSPTO_1980</i> was amplified from PsPto DNA and cloned into the BamHI sites of pUT18C and checked for correct orientation to express T18-CheY2. “gg” was introduced upstream of the start codon to ensure “in-frame” cloning; Ap <sup>r</sup> | This study        |
| pUT18C-WspR  | <i>PSPTO_1499</i> was amplified from PsPto DNA and cloned into the BamHI sites of pUT18C and checked for correct orientation to express T18-WspR. “gg” was introduced upstream of the start codon to ensure “in-frame” cloning; Ap <sup>r</sup>  | This study        |

|                                 |                                                                                                                                                                      |                                                |
|---------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------|
| pUT18C-2014                     | <i>PSPTO_2014</i> was amplified from PsPto DNA and cloned into the XmaI sites of pUTC18 and checked for correct orientation to express T18-2014; Ap <sup>r</sup>     | This study                                     |
| pUT18C-2441                     | <i>PSPTO_2441</i> was amplified from PsPto DNA and cloned into the XmaI sites of pUTC18 and checked for correct orientation to express T18-2441; Ap <sup>r</sup>     | This study                                     |
| pET28b(+)                       | N-terminal His-tagged protein expression vector; Km <sup>r</sup>                                                                                                     | Novagen, CA, USA                               |
| p2448-LBD                       | pET28b(+) harboring a His-tag in frame with 792 bp corresponding to the LBD fragment of <i>PSPTO_2448</i>                                                            | This study, (Santamaría-Hernando et al., 2022) |
| pKOSac101                       | <i>oriR6K mob</i> pKNG10 harboring a 144-bp internal deletion of <i>sacB</i> ; Sm <sup>r</sup>                                                                       | (Cerna-Vargas et al., 2019)                    |
| pKOSac101-2448                  | pKOSac101 harboring a 502-bp fragment of <i>PSPTO_2448</i> cloned at XmaI sites; Sm <sup>r</sup>                                                                     | This study, (Santamaría-Hernando et al., 2022) |
| pBBR1MCS-2::2448                | pBBR1MCS-2 plasmid harboring a the complete <i>PSPTO_2448</i> sequence and its promoter region and 266 bp upstream of its start codon; Km <sup>r</sup>               | This study, (Santamaría-Hernando et al., 2022) |
| pGEM-T easy®                    | Subcloning vector; Ap <sup>r</sup>                                                                                                                                   | Invitrogen                                     |
| pHP45Ω::Km                      | Source of the Ω::Km interposon                                                                                                                                       | (Frey & Krisch, 1985)                          |
| pHP45Ω::Sp                      | Source of the Ω::Sp interposon                                                                                                                                       | (Frey & Krisch, 1985)                          |
| pHP45Ω::Gm                      | Source of the Ω::Gm interposon                                                                                                                                       | (Frey & Krisch, 1985)                          |
| pGEM-T <i>NotI</i>              | Subcloning vector resulting from <i>NotI</i> restriction and subsequent re-ligation                                                                                  | This study                                     |
| pGEM-T <i>NotI</i> ::17070      | pGEM-T <i>NotI</i> harboring 983 bp upstream DDA_RS17070 fused to 829 bp of DDA_RS17070 cloned at <i>NotI</i> site; Ap <sup>r</sup>                                  | This study                                     |
| pGEM-T <i>NotI</i> ::12290      | pGEM-T <i>NotI</i> harboring a 2-kb overlap extension PCR fragment of DDA3937_RS12290 cloned at <i>NotI</i> site; Ap <sup>r</sup>                                    | This study                                     |
| pGEM-T <i>NotI</i> ::12165      | pGEM-T <i>NotI</i> harboring a 2-kb overlap extension PCR fragment of upstream and downstream regions of DDA3937_RS12165 cloned at <i>NotI</i> site; Ap <sup>r</sup> | This study                                     |
| pGEM-T <i>NotI</i> ::17070::ΩKm | pGEM-T <i>NotI</i> _17070 with 2.2-kb Km <sup>R</sup> cassette from pHP45Ω::Km cloned at <i>Bam</i> HI sites; Ap <sup>r</sup> , Km <sup>r</sup>                      | This study                                     |
| pGEM-T <i>NotI</i> ::12290::ΩKm | pGEM-T <i>NotI</i> ::12290 with 2.2-kb Km <sup>R</sup> cassette from pHP45Ω::Km cloned at <i>Bam</i> HI sites; Ap <sup>r</sup> , Km <sup>r</sup>                     | This study                                     |
| pGEM-T <i>NotI</i> ::12290::ΩSp | pGEM-T <i>NotI</i> ::12290 with 12-kb Sp <sup>r</sup> cassette from pHP45Ω::Sp cloned at <i>Eco</i> RI sites; Ap <sup>r</sup> , Sp <sup>r</sup>                      | This study                                     |

|                                    |                                                                                                                               |            |
|------------------------------------|-------------------------------------------------------------------------------------------------------------------------------|------------|
| pGEM-T<br><i>NotI</i> ::12165::ΩGm | pGEM-T <i>NotI</i> ::12165 with 1.6-kb Gm <sup>r</sup> cassette from<br>pHP45Ω::Gm; Ap <sup>r</sup> , Gm <sup>r</sup>         | This study |
| pBBR1MCS-2::17070                  | pBBR1MCS5 harboring a 2.1-kb fragment containing the<br><i>DDA3937_RS17070</i> gene and its promoter region; Gm <sup>r</sup>  | This study |
| pBBR1MCS-2::12290                  | pBBR1MCS-5 harboring a 1.8-kp fragment containing the<br><i>DDA3937_RS12290</i> gene and its promoter region; Gm <sup>r</sup> | This study |
| pBBR1MCS-2::12165                  | pBBR1MCS-5 harboring a 2.3-kp fragment containing the<br><i>DDA3937_RS12165</i> gene and its promoter region; Gm <sup>r</sup> | This study |

a. Rf: rifampicin, Sm: streptomycin, Km: kanamycin, Gm: gentamycin, Sp: spectinomycin, Ap: ampicillin, Tc: tetracycline

**Table 2. Primers used in this study.**

| Primer                                                               | Sequence (5'-3')                                                                                                                                | Purpose                                                |
|----------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------|
| <b>Chapter I</b>                                                     |                                                                                                                                                 |                                                        |
| cheA1-KO-XmaI-Fw<br>cheA1-KO-XmaI-Rv<br>cheA1-KO-ov1<br>cheA1-KO-ov2 | tccccccggggctcaagcaggaaagctacg<br>tccccccgggcagctggacgatctggtgag<br>gataccgtcgagctggcttccccggcggtacttcaaataag<br>gaagccagctcgacgggtatc          | Deletion of <i>PSPTO_0913</i>                          |
| cheA2-KO-ov1<br>cheA2-KO-ov2<br>cheA2-KO-XmaI-Rv<br>cheA2-KO-XmaI-Fw | cgtaacacagacctagaagccga<br>tcggcttctaaggtctgttgacgcaactggatgtggtcactgc<br>tccccccggggagcatgctgggtacatcgag<br>tccccccggggcagagcgggtgcctttgac     | Deletion of <i>PSPTO_1982</i>                          |
| cheA3-KO-ov1<br>cheA3-KO-ov3<br>cheA3-KO-XmaI-Fw<br>cheA3-KO-XmaI-Rv | gtcacgcatttgatctggcg<br>cgccagatcaaatgcgtgacgacgcgcaaggatgaagattgcc<br>tccccccgggtggccaacgaaggcaaaagc<br>tccccccgggatacctccatcaagcgcggc         | Deletion of <i>PSPTO_1497</i>                          |
| cheY1-KO-XmaI-Rv<br>cheY1-KO-XmaI-Fw<br>cheY1-KO-ov1<br>cheY1-KO-ov2 | tccccccggggcagctcaacgagatctttc<br>tccccccgggtcacggtcgtgacgtgaat<br>atgatcagggcagacactcttagccatgatctg<br>cagatcatggctactgccctgatcataaagcgg       | Deletion of <i>PSPTO_0915</i>                          |
| cheY2-KO-SpeI-Rv<br>cheY2-KO-XmaI-FW<br>cheY2-KO-ov1<br>cheY2-KO-ov2 | ggactagtagatcagcatcatccggctg<br>tccccccgggagttgatcgagcggttatgc<br>catagcgcctcaccgcatcgttgagaagtcac<br>tctcaacgatgcgggtgagggcgctatggataac        | Deletion of <i>PSPTO_1980</i>                          |
| wspR-KO-ov1<br>wspR-KO-ov2<br>wspR-KO-XmaI-Rv<br>wspR-KO-XmaI-Fw     | gaatcgtctctgttcgccgacagccggtggtt<br>cggcgaacaggagacgattctatccccgcggcagataaa<br>tccccccgggccgatatccgaacttggtgctc<br>tccccccgggtcaacgacatgcctatgg | Deletion of <i>PSPTO_1499</i>                          |
| cheWsF6-KO-XmaI-Fw<br>cheWF6-KO-XmaI-Rv<br>cheWsF6_KO-ov1            | aaacccgggcgtgctggcacacaactg<br>aaacccgggcgtcaggaatcgacagtttcg<br>tgcacatgctccgatgtccgaagaagagtgttc                                              | Deletion of <i>PSPTO_1987</i> and<br><i>PSPTO_1988</i> |

|                      |                                          |                                                                 |
|----------------------|------------------------------------------|-----------------------------------------------------------------|
| cheWsF6_KO-ov2       | tcttcggacatcggagcatgtgcagcctgata         |                                                                 |
| 0912-KO-XmaI-Fw      | tcccccggtcaatacgcgggtgctgatg             | Deletion of <i>PSPTO_0912</i>                                   |
| 0912-KO-XmaI-Rv      | ttaccggcttcgataccgct                     |                                                                 |
| 0912-KO-ov1          | agcggatcgaagccggtaacgggttactccttggaattgc |                                                                 |
| 0912-KO-ov2          | tcccccgggcggttttcgccgagtggtg             |                                                                 |
| cheA3-comp-XhoI-Fw   | aaactcgaggacgggtatcgataagcttgatatcg      | Complementation of $\Delta$ <i>cheA3</i>                        |
| cheA3-comp-NcoI-Rv   | aaaccatggtggcaatcttcaccttgcg             |                                                                 |
| cheY2-comp-XmaI-Fw   | tcccccgggcggtttgtcgtgcgtgtctg            | Complementation of $\Delta$ <i>cheY2</i>                        |
| cheY2-comp-XmaI-Rv   | tcccccgggatcagctattgacgcgttcaag          |                                                                 |
| wspR-comp-XhoI-Fw    | aaactcgaggcgcttgatggaggtattact           | Complementation of $\Delta$ <i>wspR</i>                         |
| wspR-comp-HindIII-Rv | aaaaagcttttatctgccgcgggtgata             |                                                                 |
| cheA1-T25-PstI-Fw    | aaaactgcagggatgagcattaatctcgatcaggca     | Construction of Bacterial Adenylate Cyclase Two Hybrid plasmids |
| cheA1-T25-PstI-Rv    | aaaactgcagtcagcgagaagcgggc               |                                                                 |
| CheA3-T25-BamHI-Fw   | cgcggtatccgatgacgccagatcaaatgcg          |                                                                 |
| CheA3-T25-BamHI-Rv   | cgcggtatcctcatccttgcgcgtctcc             |                                                                 |
| CheA3-NT25-BamHI-Fw  | cgcggtatccatgacgccagatcaaatgcg           |                                                                 |
| CheA3-NT25-BamHI-Rv  | cgcggtatccccctccttgcgcgtctcc             |                                                                 |
| cheY1-T18-BamHI-Fw   | cgcggtatccatggctaagagtgtattggtg          |                                                                 |
| cheY1-T18-BamHI-Rv   | cgcggtatccccgggcagaatcagtttgag           |                                                                 |
| cheY1-T18C-BamHI-Fw  | cgcggtatccgatggctaagagtgtattggtg         |                                                                 |
| cheY1-T18C-BamHI-Rv  | cgcggtatcctcagggcagaatcagtttg            |                                                                 |
| cheY2-T18-BamHI-Fw   | cgcggtatccatgaaatcctcatcgttgatg          |                                                                 |
| cheY2-T18-BamHI-Rv   | cgcggtatccccgctattgacgcgttcaag           |                                                                 |
| cheY2-T18C-BamHI-Fw  | cgcggtatccgatgaaatcctcatcgttgatg         |                                                                 |
| cheY2-T18C-BamHI-Rv  | cgcggtatcctcagctattgacgcgttc             |                                                                 |
| wspR-T18-BamHI-Fw    | cgcggtatccatggatgacctgcaggcgg            |                                                                 |
| wspR-T18-BamHI-Rv    | cgcggtatccccgccggcagccacctgatt           |                                                                 |
| wspR-T18C-BamHI-Fw   | cgcggtatccgatggatgacctgcaggcgg           |                                                                 |
| wspR-T18C-BamHI-Rv   | cgcggtatcctcagccggcagccacc               |                                                                 |
| fliM-T18-BamHI-Fw    | cgcggtatcctcacatggccgtg                  |                                                                 |
| fliM-T18-BamHI-Rv    | cgcggtatccggacgtcgcgtatgggttc            |                                                                 |
| fliM-T18C-BamHI-Fw   | cgcggtatcctcaactgcgcgtatggg              |                                                                 |
| fliM-T18C-BamHI-Rv   | cgcggtatcctcacatggccgtg                  |                                                                 |
| 0912-T18-XmaI-Fw     | tcccccggggatgaaatggttctacgatctga         |                                                                 |
| 0912-T18-XmaI-Rv     | tcccccgggggaaacgggtgaattccga             |                                                                 |
| 0912-T18C-XmaI-Fw    | tcccccggggggatgaaatggttctacgatctga       |                                                                 |
| 0912-T18C-XmaI-Rv    | tcccccggggtcagaaacgggtgaattcc            |                                                                 |
| 2480-T18-XmaI-Fw     | tcccccggggatgaaaaatctgggcttcagcaag       |                                                                 |
| 2480-T18-XmaI-Rv     | tcccccgggggatccggaagctgccgac             |                                                                 |
| 2480-T18C-XmaI-Fw    | tcccccggggggatgaaaaatctgggcttcagcaag     |                                                                 |
| 2480-T18C-XmaI-Rv    | tcccccggggtcagatccggaagctgcc             |                                                                 |
| 2014-T18-XmaI-Fw     | tcccccggggatgcgcaacaaccagcc              |                                                                 |
| 2014-T18-XmaI-Rv     | tcccccgggggcgattaaatcgttcacaagc          |                                                                 |
| 2014-T18C-XmaI-Fw    | tcccccggggggatgcgcaacaaccagcc            |                                                                 |

|                   |                                                  |                                                                       |
|-------------------|--------------------------------------------------|-----------------------------------------------------------------------|
| 2014-T18C-XmaI-Rv | tccccccgggtcagcgattaaatcgttccaca                 | Construction of Bacterial Adenylate Cyclase Two Hybrid plasmids       |
| 2441-T18-XmaI-Fw  | tccccccgggatgtcgaccaccacagaaag                   |                                                                       |
| 2441-T18-XmaI-Rv  | tccccccggggtgcaggtgtccgatgg                      |                                                                       |
| 2441-T18C-XmaI-Fw | tccccccgggggatgtcgaccaccacagaaag                 |                                                                       |
| 2441-T18C-XmaI-Rv | tccccccgggtcatgcaggtgtccgatg                     |                                                                       |
| 1493-T18-XmaI-Fw  | tccccccgggatgaagaactggaccttgcg                   |                                                                       |
| 1493-T18-XmaI-Rv  | tccccccgggggaccttgaaacgcgacacg                   |                                                                       |
| 1493-T18C-XmaI-Fw | tccccccgggggatgaagaactggaccttgcg                 |                                                                       |
| 1492-T18C-XmaI-Rv | tccccccgggtcagaccttgaaacgcgac                    |                                                                       |
| Chapter II        |                                                  |                                                                       |
| 2448XmaI-Fw       | aaccggggcctacgtttcttacggtc                       | Construction of PsPto- <i>pscC</i> mutant                             |
| 2448XmaI-Rv       | ttccggggtgatcaggccgataaagt                       |                                                                       |
| 2448Comp-Fw       | aaaagaattcgcgctgcggcacgtcc                       | Construction of plasmid for PsPto- <i>pscC</i> mutant complementation |
| 2448Comp-Rv       | tttgaattcctacgctccagcaggaatg                     |                                                                       |
| rpoD-Fw           | cggcatcgacatgaataccg                             | RT-qPCR                                                               |
| rpoD-Rv           | gcgcagtgcccttggttc                               |                                                                       |
| gabT1-Fw          | actctcatctgctccgccag                             |                                                                       |
| gabT1-Rv          | ccgtcgacgtcccacagttca                            |                                                                       |
| gabT2-Fw          | tccctgatgcaacgccgc                               |                                                                       |
| gabT2-Rv          | cgatgaattcacgaccttccacgtc                        |                                                                       |
| gabT3-Fw          | tccacagcatgaccttgctccac                          |                                                                       |
| gabT3-Rv          | gccccagattgagcacgcc                              |                                                                       |
| gabD1-Fw          | agaccccgacctgctggtg                              |                                                                       |
| gabD1-Rv          | cgatcacttcgccggtcgc                              |                                                                       |
| gabP-Fw           | tagcgtgagtggcgtcagtca                            |                                                                       |
| gabP-Rv           | acgatctcggtgcccatgaacg                           |                                                                       |
| hrpL-Fw           | catcttcgtccgccgtattc                             |                                                                       |
| hrpL-Rv           | cgcactggagaatgtcatccac                           |                                                                       |
| avrPto-Fw         | gacatgcagcataggtacatgac                          |                                                                       |
| avrPto-Rv         | gcttcgcgcatgtcactc                               |                                                                       |
| Chapter III       |                                                  |                                                                       |
| 17070-ov1-EcoRI   | gttaccatcaatagttatttgaattctatatcgtgagaatggaagggc | Mutation of <i>DDA_RS17070</i>                                        |
| 17070-ov2-EcoRI   | gaattcaaataactattgatggaacgatcgatcggtttctgcaacg   |                                                                       |
| 17070-NotI-Fw     | aagcggccgcgatctgggcaatggagtcat ta                |                                                                       |
| 17070-NotI-Rv     | aagcggccgcctgggggtctatcgccaaatc                  |                                                                       |
| 12290-NotI-Fw     | aagcggccgcgcccttgctcagaacatta                    | Mutation of <i>DDA_RS12290</i>                                        |
| 12290-NotI-Rv     | aagcggccgcccgtattctgatgggtcaatt                  |                                                                       |
| 12290-ov1-BamHI   | ccggcctggtatttggatcctgcgatattgaagggtagagca       |                                                                       |
| 12290-ov2-BamHI   | ggatccaaataaccaggccggtggaacagattctcgaacag        |                                                                       |
| 12165_ov1-EcoRI   | aaggcctgttgaattcactggctgattgttgcgc               | Mutation of <i>DDA_RS12165</i>                                        |
| 12165_ov2-EcoRI   | atcagccagtgaattcaacaggccttgacgtattc              |                                                                       |
| 12165-NotI-Rv     | aaagcggccgcggtggcacatgcgattatc                   |                                                                       |
| 12165-NotI-Fw     | aaaagcggccgcgttctgaccgacatcgatgc                 |                                                                       |

## Results



## **1. CHAPTER I**

**Chemosensory systems interact to shape relevant traits for  
bacterial plant pathogenesis**



## 1.1. Introduction

Out of the different chemosensory systems, the chemotaxis system is the most well-known, which controls the motility towards attractants or away from repellents. However, as mentioned earlier, these systems appear to be involved not only in the control of flagellar motility but also in the regulation of other cellular functions such as type IV pilus-based motility, biofilm formation, or the control of cyclic diguanosine monophosphate (c-di-GMP) and cyclic adenosine monophosphate (c-AMP) second messenger levels (Fulcher et al., 2010; Hickman et al., 2005; Whitchurch et al., 2004).

Chemosensory systems assemble into large structures called chemotaxis or chemosensory arrays. Briefly, chemoreceptors (CRs) associate together with CheA and CheW to form the arrays depending on their physical length, which is determined greatly by their heptad class. This means that CRs with different LBDs, and probably different specificities, belonging to the same sensory domain length group, can arrange together facilitating signal integration (Parkinson et al., 2005).

In bacteria with multiple chemosensory systems, the spatial segregation of arrays formed by each system, comprising different CRs, would be advantageous for preventing interference among different functions (Kentner & Sourjik, 2006). However, there are experimental data regarding the existence of crosstalk between chemosensory systems (Berne & Brun, 2019; Huang et al., 2019; Liu et al., 2018b; O’Neal et al., 2019), thus raising the question of how these systems are structurally and functionally separated, and whether this occurs at the level of array formation (Yang & Briegel, 2020).

As it has been previously mentioned, *Pseudomonas syringae* pv. tomato DC3000 (PsPto) harbors 49 CRs and four gene clusters that encode four main chemosensory systems encoded in independent gene clusters: cluster I (F6 chemosensory system), cluster II (F8 chemosensory system), cluster III (ACF chemosensory system), and cluster IV (Tfp chemosensory system). Previous results determined that cluster II is responsible for chemotaxis (Cerna-Vargas et al., 2019), as a defective *cheA2* mutant lost chemoattraction to several compounds. Moreover, this mutant also displayed an altered phenotype in the levels of the second messenger c-di-GMP suggesting a crosstalk between chemosensory systems (Cerna-Vargas et al., 2019). In addition, it has been shown that a PsPto *cheA2* mutant is impaired in virulence in tomato plants (Clarke et al., 2016).

Although previous studies on PsPto and *P. syringae* pv. tabaci have reported the analysis of phenotypes associated with mutations in the F6 and F8 chemosensory systems present in these bacteria (Clarke et al., 2016; Tumewu et al., 2021), the way the different chemotaxis pathways connect and interplay to resolve adaptation to plant host is not well described.

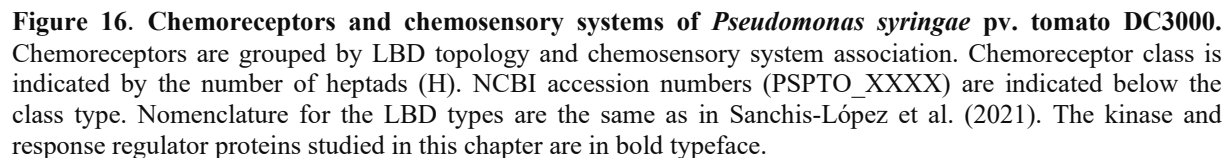
In this work, we propose an assignment of the 49 CRs to the one of the four chemosensory systems found in this bacterium. Moreover, we show, for the first time, the visualization of two chemosensory arrays in this bacterium and analyze the function, crosstalk, and interaction between components of the main PsPto chemosensory systems F6, F8 and ACF in relation with pathogenicity traits.

## 1.2. Results

### 1.2.1. Assigning chemoreceptors to chemosensory systems in PsPto

Most chemotaxis genes in *P. syringae* pv. tomato DC300 (PsPto) are arranged in four gene clusters (cheI-cheIV) called chemosensory systems. Additional chemotaxis genes are located across the genome with no particular organization. Main clusters were initially numbered according to their homology with other *Pseudomonas* species (*Pseudomonas aeruginosa* PAO1 and *Pseudomonas putida* KT2440) (Sampedro et al., 2014) regarding genomic organization. Genes in the clusters were annotated before the cluster numbering, and therefore numbering of genes differs from the numbering of the chemosensory system they belong to (Figure 16).

All chemotaxis genes and chemoreceptor genes from PsPto were retrieved using MiST 4.0 database. MiST 4.0 (mistdb.com) associates chemotaxis genes to specific chemosensory categories depending on gene orthology, protein structure, and experimental evidence in related bacteria (Gumerov et al., 2020). The MiST4.0 predicts that PsPto has 49 chemoreceptors (41 from the 40H class, 1 from the 36H class, 1 from the 34H class, and 6 from the 24H class). The four PsPto chemosensory systems are categorized as F6, F8, ACF, and Tfp (Figure 16).



In bacteria with a single chemotaxis cluster the 24H receptor class has been assigned to work with F1, F3, F5, F6, and F7 systems (Ortega et al., 2017). Since only the F6 system is present in PsPto, we assign all six 24H receptors to the F6 system (cluster I). The F6 system is known to also work with 40H receptors (Ortega et al., 2017), therefore we assign all 40H chemoreceptors to the F6 system, except for two chemoreceptors, PSPTO\_1493 and PSPTO\_5031. PSPTO\_1493 exhibits synteny and shares 69.2% identity with the *wspA* gene of PAO1, with a genomic location in the ACF cluster. PSPTO\_5031 exhibits synteny and shares 77.4% identity with the *pilJ* gene of PAO1, encoded in the Tfp cluster. Therefore, we assign PSPTO\_1493 and PSPTO\_5031 to the ACF and Tfp system, respectively (Figure 16 and Table S1). Moreover, experimental data is available on the homologues chemoreceptors of PAO1 showing their function in association with these classes of chemosensory systems (Kearns et al., 2001; O'Connor et al., 2012). PSPTO\_0912 is the only receptor belonging to the 34H class, which is a minor class of chemoreceptors. PSPTO\_0912 is encoded in the F8 gene cluster (cluster II) suggesting its function in association with this system. Moreover, the assignment of 34H chemoreceptors to the F8 system has been previously proposed in other organisms (Fortier et al., 2022; Ortega et al., 2020) (Figure 16 and Table S1). PSPTO\_2441 is the only chemoreceptor belonging to the 36H class, which have been mostly assigned to the F7, F1, F3 and Tfp classes (Ortega et al., 2017). From these systems, only Tfp is present in PsPto, therefore we could assign PSPTO\_2441 to the Tfp class (Figure 16 and Table S1). However, PSPTO\_2441 is a cytoplasmic CR harboring four PAS domains. Most of the CRs with PAS domains have been assigned to the F6 system, therefore a definitive assignment cannot be performed for this CR.

An experimental validation of this assignment was attempted by performing Bacterial Adenylate Cyclase Two Hybrid (BACTH) assays. First, we chose chemoreceptors that were representative of each heptad class and presumably associated with a chemosensory system, namely PSPTO\_0912 (34H, F8), PSPTO\_2480 (40H, F6), PSPTO\_1493 (40H, ACF), PSPTO\_2014 (40H, F6), and PSPTO\_2441 (36H, unknown). PSPTO\_5031 was not included because it falls out of the scope of this investigation. We determined protein interaction between each chemoreceptor and each CheA kinase from the F6 (CheA2), F8 (CheA1), and ACF (CheA3) chemosensory systems (Figure 17).

Surprisingly, PSPTO\_0912 and PSPTO\_2014 showed interaction with all three kinases. Moreover, while PSPTO\_2441 showed interaction with CheA2 (F6) and CheA3 (ACF), PSPTO\_2480 only showed interaction with CheA2. Finally, we were unable to detect interaction between PSPTO\_1493 and any of the kinases.

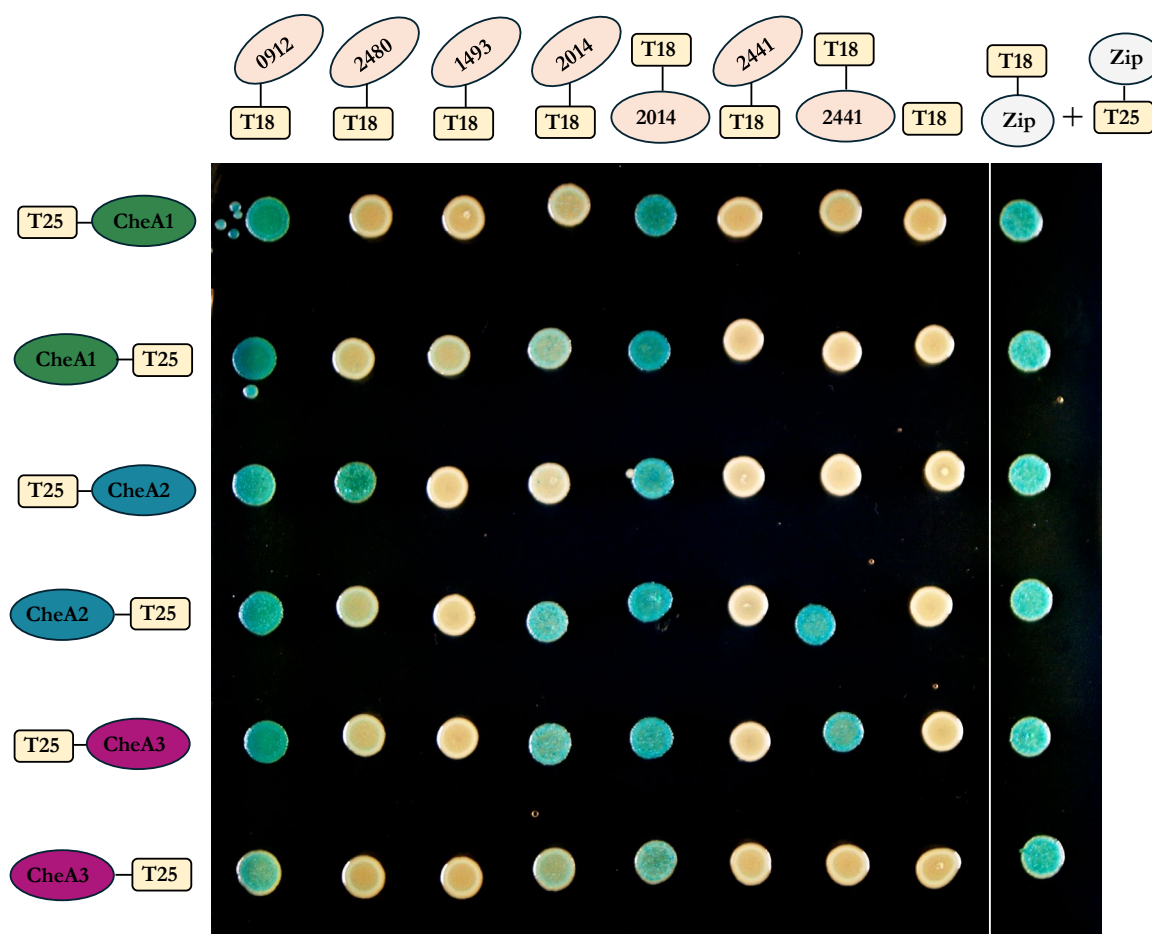


**Figure 17. Bacterial adenylate cyclase two hybrid assays between CheAs and chemoreceptors from PsPto.** Experiments were conducted in triplicate with three technical replicates each. For better visualization, only one replicate is shown for each interaction. A schematic representation of proteins cloned at N-terminal or C-terminal of the T25 or T18 adenylate cyclase fragment is presented. CheA1, CheA2, and CheA3 were tested against the T18 fragment of the adenylate cyclase as a negative control. Interaction between leucine zipper of GCN4 in frame with the T25 and T18 fragment of the adenylate cyclase was used as a positive control.

### 1.2.2. Cryo-Electron tomography reveals two distinct chemotaxis arrays in PsPto

From all the chemosensory system types found in PsPto, only the F6 type has been successfully imaged before in other bacteria using cryo-electron microscopy (Ortega et al., 2020). To our knowledge, F8, ACF and Tfp systems have not been previously imaged. In the case of ACF and Tfp systems, it is still unknown if they assemble clusters large enough to be visible with cryo-electron microscopy. Thus, we focused on building structural models for the F6 and F8 system using two chemoreceptor sequences from class 40H (PSPTO\_2480) and 34H (PSPTO\_0912). In the case of the 34H chemoreceptor, we measured 20 nm from the I215 at the base of the transmembrane region to the G380 at the tip of the receptor. In the case of the 40H chemoreceptor, we measured 23 nm from the

M301 to the G488 at the tip of the chemoreceptor. Thus, we predicted the F8 system to have a height of 20 nm and the F6 system a height of 23 nm.

We used cryo-electron tomography to visualize and characterize the chemoreceptor arrays in PsPto (Figure 18). We were able to visualize two distinct arrays in wild-type (WT) cells of 20 and 23 nm in height. This is consistent with our predicted measurements, thus identifying the arrays as F8 and F6 systems, respectively. In *Escherichia coli*, the absence of CheA kinase impedes the formation of the chemotaxis array. Since CheA1 is predicted to take part in the F8 array, and CheA2 in the F6 array, we also imaged cells from  $\Delta cheA1$  and  $\Delta cheA2$  strains to confirm which kinase forms each array.  $\Delta cheA1$  cells only showed F6 arrays, which indicates that CheA1 participates in the formation of F8 arrays. Surprisingly,  $\Delta cheA2$  showed both F6 and F8 arrays (Figure 18D, Table S2).

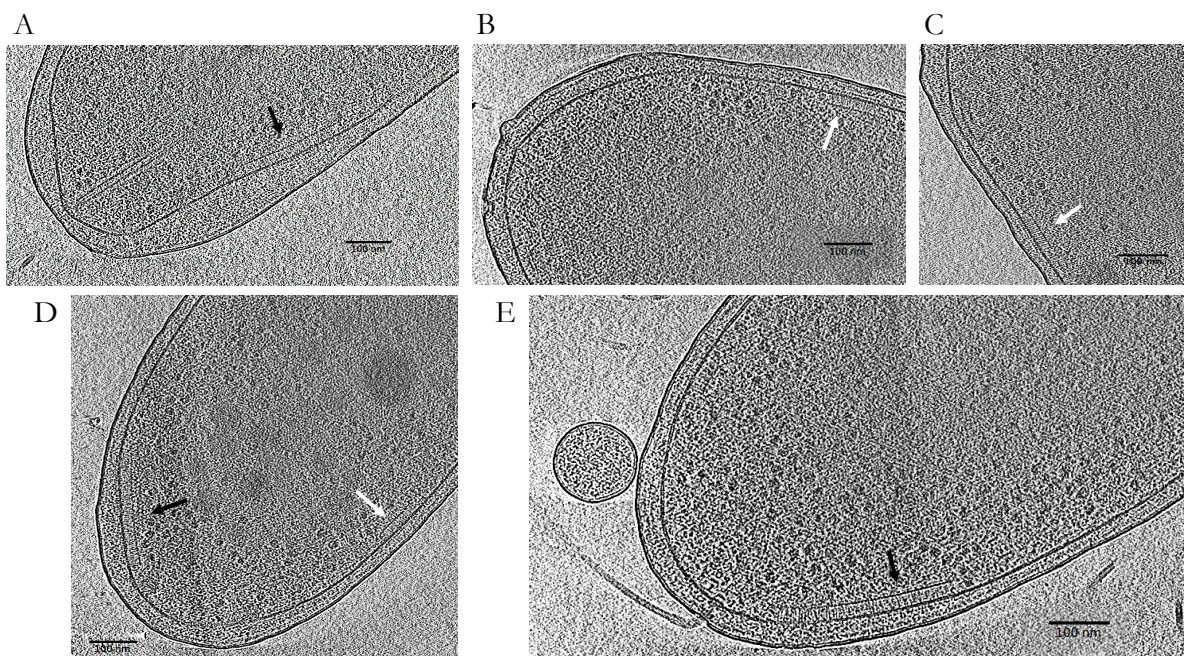


**Figure 18. Cryo-electron microscopy of WT,  $\Delta cheA1$ , and  $\Delta cheA2$  cells from PsPto.** Black arrows point to chemosensory arrays of type F6, and white arrows point to chemosensory arrays of type F8. (A) WT with F6 array. (B) WT with F8 array. (C)  $\Delta cheA2$  with F8 array. (D)  $\Delta cheA2$  with F6 and F8 arrays. (E)  $\Delta cheA1$  with F6 array.

### 1.2.3. Chemotaxis is impaired in $\Delta cheA2$ but not in $\Delta cheA1$ and $\Delta cheA3$ PsPto mutant strains

To unveil the role of the different PsPto chemosensory systems in the control of flagellar motility driven by the chemoperception process, we performed capillary based chemotaxis assays against casamino acids, which are strong chemoattractants for *Pseudomonas*. We tested this phenotype in the  $\Delta cheA1$ ,  $\Delta cheA2$  and  $\Delta cheA3$  mutant strains, altered in the kinase of each chemosensory system.

Results showed that only the F6 system is involved in the control of chemotaxis in PsPto (Figure 19). These results are in line with the previous reported involvement of this system in amino acid perception (Cerna-Vargas et al., 2019).

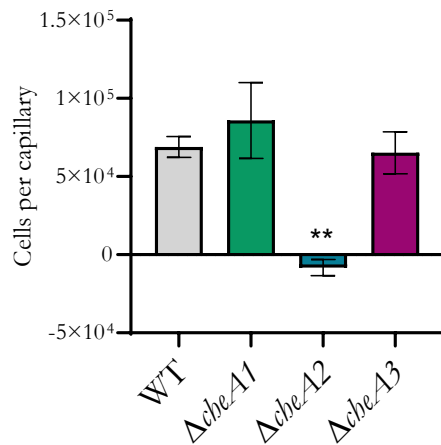


**Figure 19. Chemotaxis capillary assays of WT and histidine kinase mutants against 0.1 mM casamino acids.** The data have been corrected with the number of cells that swam into buffer-containing capillaries. Shown are means and standard errors from three independent experiments with three technical replicates each. Values that are significantly different are indicated by asterisks (\*\* $p < 0.01$ ).

#### 1.2.4. Chemosensory systems regulate swimming motility

To assess the role of the PsPto chemosensory systems in chemotaxis related motility, we performed soft agar swimming assays in strains lacking *cheA* or the corresponding response regulator in each chemosensory system (Figure 20). To account for specific media variation, we performed the chemotaxis swimming experiments in rich media, M9 minimal media supplemented with 10 mM citrate as carbon source, and in *hrp*-inducing media (HIM) (Huynh et al., 1989), a minimal media that mimics the plant apoplast. All strains lacking *cheA* showed swimming defects, but deletion of *cheA2* completely abolished motility in all media tested. Regarding the response regulator mutant strains, Δ*cheY2* displays the same swimming phenotype as Δ*cheA2* in all media tested and Δ*wspR* displays a similar phenotype as Δ*cheA1* and Δ*cheA3*, showing a decreased swimming motility. However, Δ*cheY1* showed similar motility phenotype to the WT strain in all media tested. Swimming was partially restored in Δ*cheA2* complemented strain and totally restored in Δ*cheY2* complemented strain (Figure S1AB). Swimming complementation of Δ*cheA1*, Δ*cheA3* and Δ*wspR* strains was not observed (Figure S1CDE).

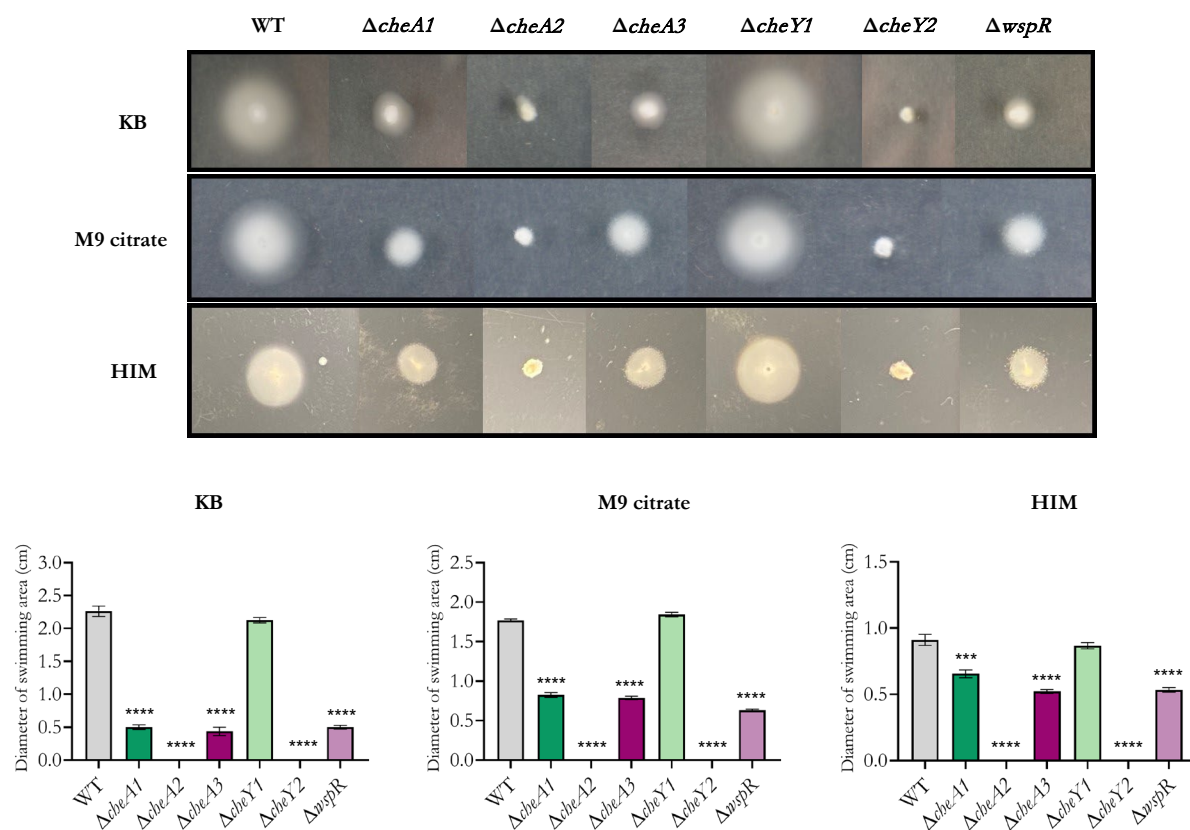


**Figure 20. Swimming motility assays of PsPto histidine kinase and response regulator mutants in different media.** Shown are means and standard errors from three independent experiments with four technical replicates each. Values that are significantly different are indicated by asterisks (\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

As it has been previously mentioned, it has been proven that CheY-type response regulators bind to the flagellar motor protein FliM to modulate flagellar rotation (Toker & Macnab, 1997). Based on the two opposite motility phenotypes observed in the two PsPto  $\Delta cheY$  mutants, we carried out BACTH assays to test interactions between PsPto FliM and the two CheY proteins. Results showed that CheY2 interacts with FliM while no binding was detected between CheY1 and FliM (Figure 21).

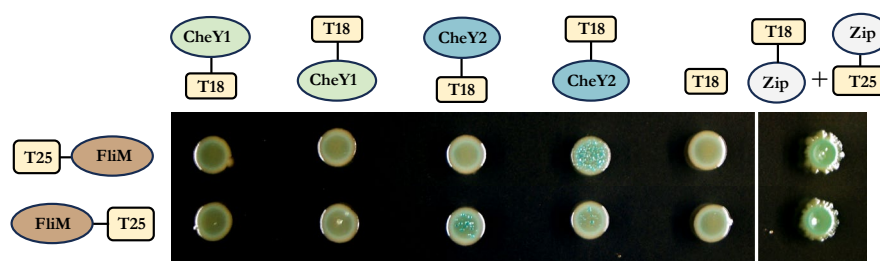


**Figure 21. Bacterial adenylate cyclase two hybrid assays between FliM and the CheY1 and CheY2 proteins from PsPto.** Experiments were conducted in triplicate with three technical replicates each. For better visualization, only one replicate is shown for each interaction. A schematic representation of proteins cloned at N-terminal or C-terminal of the T25 or T18 adenylate cyclase fragment is presented. FliM was tested against the T18 fragment of the adenylate cyclase as a negative control. Interaction between leucine zipper of GCN4 in frame with the T25 and T18 fragment of the adenylate cyclase was used as a positive control.

Overall, these results indicate that CheA2 and CheY2 are essential for chemotaxis driven motility, but CheA1, CheA3, and WspR also contribute to this trait. In contrast, CheY1 does not seem to be involved in the regulation of flagellar dependent motility.

### 1.2.5. Chemosensory systems also play a role in modulating twitching motility

Observing the altered swimming behavior in chemosensory systems mutant strains, we opted to evaluate their ability to execute an alternative form of motility (Figure 22). Twitching is a type of motility derived from contact with surfaces. In our experiments, the  $\Delta cheA1$ ,  $\Delta cheA2$ ,  $\Delta cheA3$  and the  $\Delta wspR$  mutant strains displayed a larger twitching area than the WT strain. In contrast,  $\Delta cheY2$  displayed a smaller twitching area than the WT strain, and no differences were observed in the case of  $\Delta cheY1$  compared to the WT.

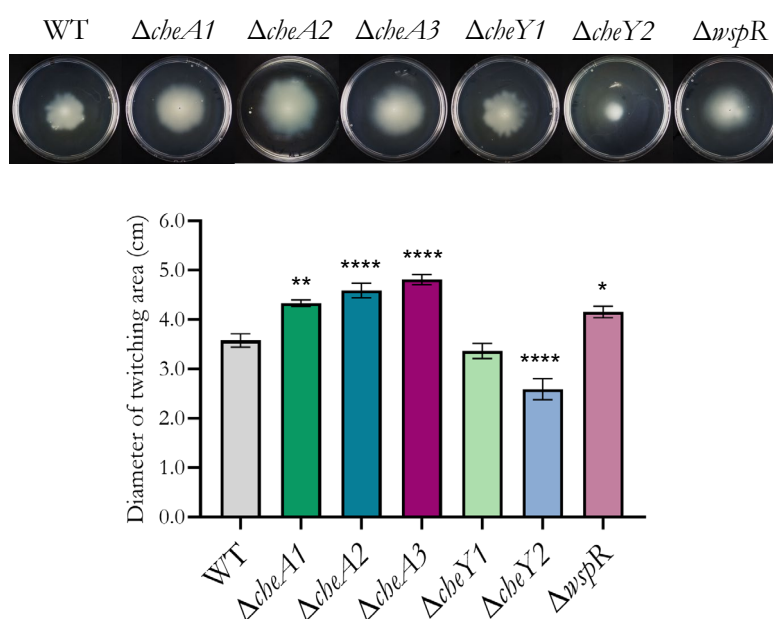


**Figure 22. Twitching motility assays of PsPto histidine kinase and response regulator mutants.** Shown are means and standard errors from three independent experiments with three technical replicates each. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ ).

### 1.2.6. Chemosensory systems contribute to biofilm-related phenotypes

As motility and biofilm formation are tightly regulated traits closely intertwined with each other, we decided to assess the biofilm formation ability in our chemosensory systems mutants (Figure 23). Attachment assays were performed using the crystal violet method described previously

(Chakravarthy et al., 2017) (Figure 23A).  $\Delta cheA1$ ,  $\Delta cheA2$ , and  $\Delta wspR$  showed a significant increase in attachment when compared to the WT. Contrarily,  $\Delta cheY1$  and  $\Delta cheY2$  attached significantly less than the WT strain. However, a  $cheA3$  deletion did not cause any attachment alteration in this assay.

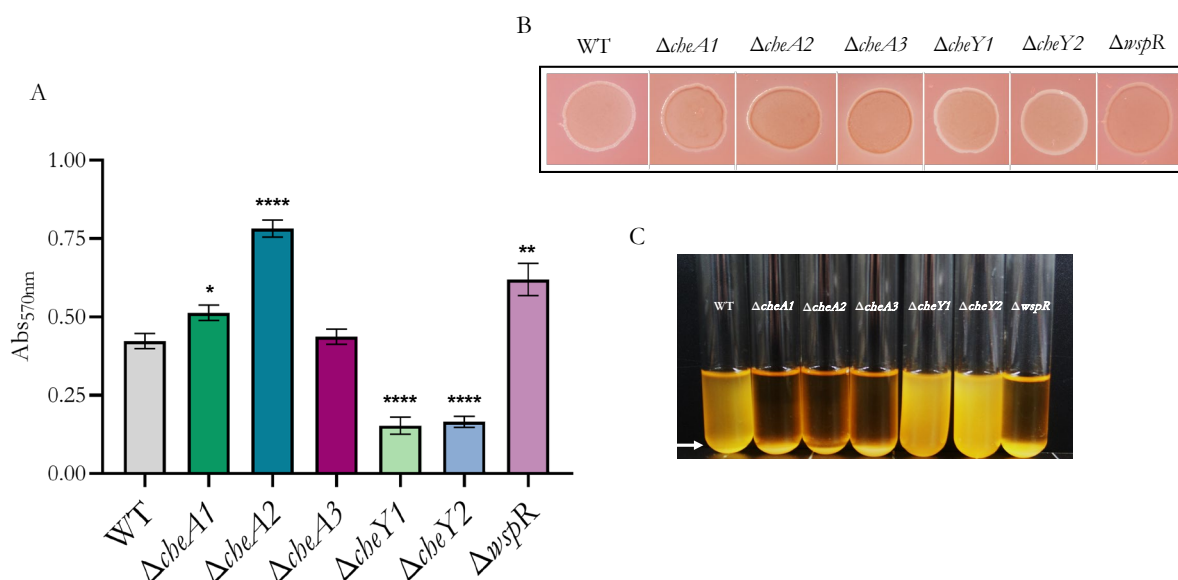


**Figure 23. Biofilm-related traits.** (A) Crystal violet attachment assay. Shown are means and standard errors from three independent experiments with six technical replicates each. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ ). (B) Representative image of the Congo red uptake by colonies grown on M9 citrate plates. Three independent experiments were conducted with six technical replicates each. (C) Flocculation assays of the WT and mutant strains. Three independent experiments were conducted with three technical replicates each. White arrow points to the sedimentation area.

We decided to analyze the Congo red uptake ability, which is related with exopolysaccharide (EPS) production and indirectly with biofilm formation (Jones & Wozniak, 2017) (Figure 23B). First, we observed that  $\Delta cheA1$ ,  $\Delta cheA2$ ,  $\Delta cheA3$ , and  $\Delta wspR$  presented a higher Congo red uptake ability than the WT. In contrast,  $\Delta cheY1$  and  $\Delta cheY2$  showed a similar Congo red uptake to the WT strain. Flocculation phenotype in liquid media is also related to EPS production, and we also observed that liquid cultures of  $\Delta cheA1$ ,  $\Delta cheA2$ ,  $\Delta cheA3$ , and  $\Delta wspR$  flocculated overnight when left static at room temperature, whereas the WT flocculated partially, and  $\Delta cheY1$  and  $\Delta cheY2$  remained homogeneously distributed across the liquid media (Figure 23C). We also analyzed the colony morphology and texture in rich media, which has been also related to the biofilm formation ability (Ha & O'Toole, 2015) (Figure S2). We could observe how the inner colony areas of  $\Delta cheA1$ ,  $\Delta cheA2$ ,  $\Delta cheA3$ , and  $\Delta wspR$  exhibited more pronounced wrinkling compared to the WT. In contrast,  $\Delta cheY1$  and  $\Delta cheY2$  displayed a smooth colony morphology.

### 1.2.7. Bacterial two-hybrid assays reveal unusual interaction partners

The above-described results point to a possible crosstalk between the three chemosensory systems analyzed in this work (F6, F8, and ACF). Canonically, we would expect the kinase CheA2 to interact with the response regulator CheY2 (F6 system), CheA1 to interact with CheY1 (F8 system), and CheA3 to interact with WspR (ACF system). BACTH assays were performed to check interactions among the three kinases and the three response regulators. Results showed that, as expected, CheA2 and CheA3 interact with their canonical response regulators CheY2 and WspR. Surprisingly, we were unable to detect the interaction between CheA1 and CheY1 response regulator. Moreover, a clear interaction was observed in the case of both CheA1 and CheA2 with the response regulator WspR (Figure 24).

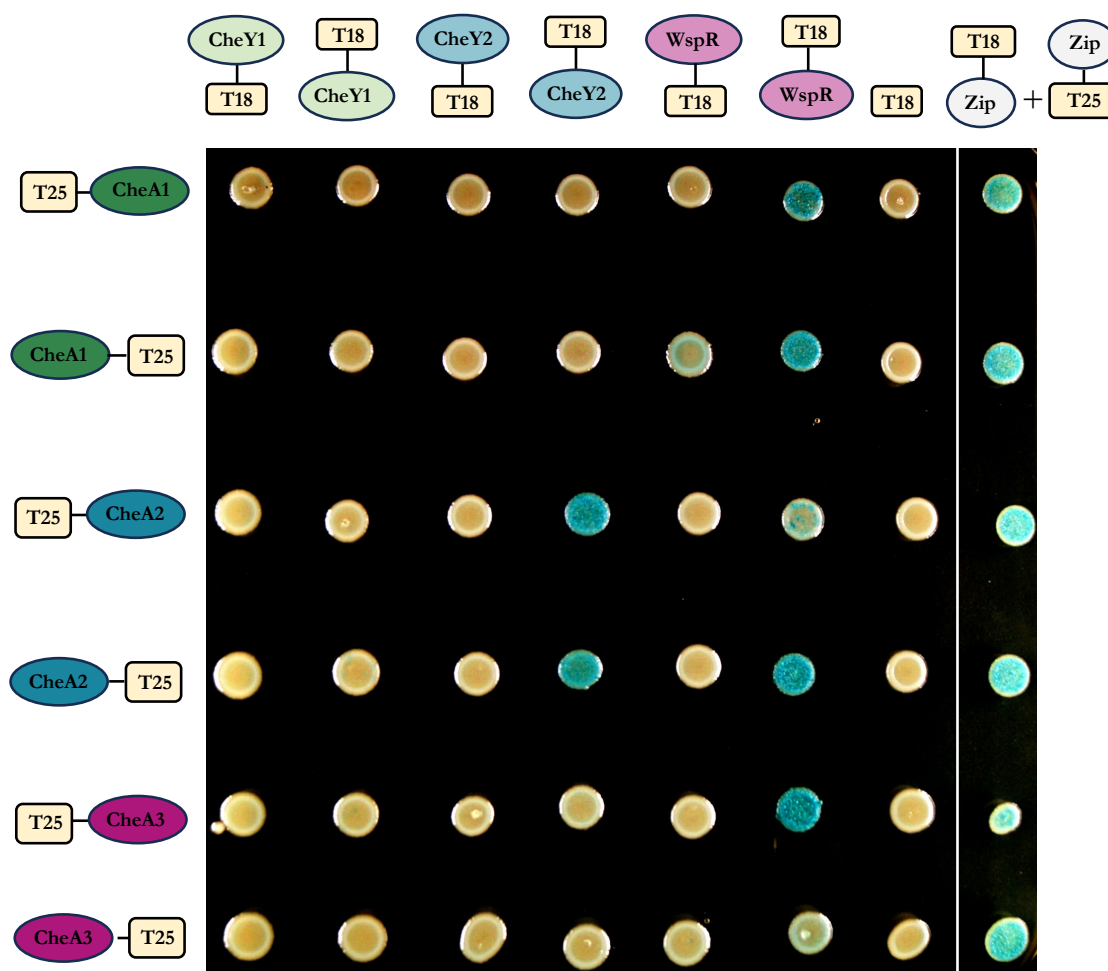


**Figure 24. Bacterial adenylate cyclase two hybrid assays between CheAs and response regulators from PsPto.** Experiments were conducted in triplicate with three technical replicates each. For better visualization, only one replicate is shown for each interaction. A schematic representation of proteins cloned at N-terminal or C-terminal of the T25 or T18 adenylate cyclase fragment is presented. CheA1, CheA2, and CheA3 were tested against the T18 fragment of the adenylate cyclase as a negative control. Interaction between leucine zipper of GCN4 in frame with the T25 and T18 fragment of the adenylate cyclase was used as a positive control.

### 1.2.8. Chemosensory systems are necessary for an optimal plant entry to incite infection

We tested the ability of the PsPto chemosensory mutant strains to infect tomato plants. We used different inoculation methods, the spray-inoculation approach, which mimics natural entry conditions that bacteria face in the phyllosphere, and the infiltration method, in which the pathogen is placed inside the plant. Bacterial populations of mutant strains were reduced compared to that of the WT strain when cells were spray-inoculated. However, this difference was abolished when the strains were inoculated by infiltration (Figure 25). Nevertheless, we could observe how syringe-inoculated tomato leaves with  $\Delta cheA3$  and  $\Delta wspR$  mutant strains displayed a decreased necrotic area than when inoculated with the WT strain (Figure S3). These findings demonstrate how all three chemosensory systems play a key role during the early stages of the infection of tomato plants. Furthermore, the ACF system also seems to be involved in symptom development.

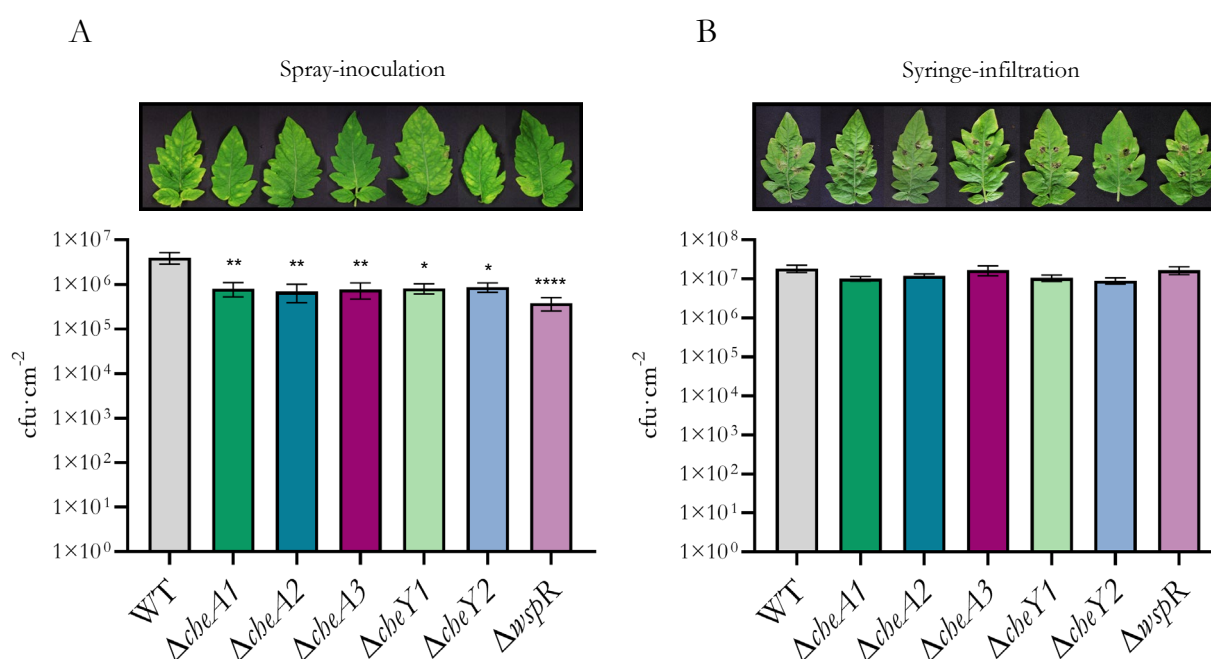


**Figure 25. Tomato virulence assays with two inoculation methods.** (A) Spray-inoculated virulence assays. Shown are means and standard errors of bacterial populations of strains at 6 days postinoculation (dpi) after spray-inoculation ( $10^8$  cfu·ml<sup>-1</sup>) (B) Syringe-infiltration virulence assay. Shown are means and standard errors of bacterial populations of strains at 3 dpi after syringe-infiltration ( $3 \times 10^4$  cfu·ml<sup>-1</sup>). Three independent experiments were conducted with three technical replicates each. In each experiment, three plants were analyzed by sampling five 1-cm-diameter leaf discs per plant. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ ).

### 1.3. Discussion

Many bacteria have evolved more than one chemosensory system which arrange independently, and it has been proposed that this cluster-based scaffolding allows the spatial separation of chemotaxis pathways putatively involved in specific cellular functions (Kentner & Sourjik, 2006).

PsPto has four main chemosensory clusters, namely F6, F8, ACF, and Tfp systems, but many questions remain to be answered regarding the specific functions they control. We have assigned the 49 chemoreceptors of PsPto to one of the four main chemosensory clusters. The assignment is based solely in a deductive process based on previous data and assignments in other organisms (Ortega et al., 2020). Even though we attempted to validate this assignment through protein-protein interaction assays, further experimental data will be necessary to confirm or refute our assignment.

Most of the 40H chemoreceptors have been assigned to the F6 system. However, the 40H chemoreceptors PSPTO\_1493 and PSPTO\_5031 are proposed to be assigned to the ACF and Tfp systems respectively. This assignment is based on both genomic context and experimental data on homologous chemoreceptors of PAO1 (Kearns et al., 2001; O'Connor et al., 2012). Unfortunately, we were not able to validate the assignment of PSPTO\_1493, as we could not detect any interaction with the CheA3 kinase. BACTH constructs are made with the T25 and T18 regions both at N- and C-terminal to avoid possible unstable protein fusions when cloned in one orientation only. Unfortunately, we were unable to obtain the genetic constructs for BACTH assays in both orientations, only succeeding in the construction of a fusion protein at C-terminal. Therefore, negative results should be taken with care.

Regarding the assignment of the remaining 40H chemoreceptors to the F6 system, we could detect interaction between the PSPTO\_2480 and the CheA2 kinase (F6), which could validate this assignment. However, PSPTO\_2014 (also 40H) showed interaction with all three kinases. PSPTO\_2014 is structurally homologous to *E. coli* Aer, with a PAS-domain type of LBD facing the cytoplasm instead of the periplasm. Although it belongs to the 40H class, and therefore it was assigned to the F6 system, we cannot exclude its interaction with the remaining chemosensory systems.

Regarding PSPTO\_0912, the only 34H class chemoreceptors of PsPto, we proposed its assignment to the F8 system based on its genomic location. Moreover, it is the only chemoreceptor of PsPto containing a C-terminal pentapeptide, a domain known to bind to specific CheRs that contain a characteristic GXX insertion (García-Fontana et al., 2014). The only CheR in PsPto which presents this feature is also encoded in the F8 cluster (*PSPTO\_0910*), which supports the assignment of PSPTO\_0912 to this system. Moreover, we can confidently say that PSPTO\_0912 is forming the F8 chemosensory array in PsPto, since the predicted height of an array formed by PSPTO\_0912

chemoreceptors coincided with the actual measured height for the F8 system in the obtained cryotomograms. However, this chemoreceptor showed interaction with all three kinases in the BACTH assays, meaning it could feed all three chemosensory systems. Therefore, we cannot rule out that PSPTO\_0912 is also signaling through the F6 or ACF systems.

PSPTO\_2441 is the only chemoreceptor of the 36H class. The chemoreceptors from this heptad class have been assigned to Tfp systems in other bacteria. However, as chemoreceptors of 36H and 40H class tend not to cluster together (Briegel et al., 2009) and experimental data supports the assignment of PSPTO\_5031 (40H) to the Tfp system (Kearns et al., 2001), we could speculate that PSPTO\_2441 is working with the F6 system instead. In fact, the F6 system has been proposed to cluster chemoreceptors from different heptad classes (Ortega et al., 2017). PSPTO\_2441 is a cytoplasmic chemoreceptor which contains 4 consecutive PAS\_3 domains linked to an MCP signal domain. All 24H chemoreceptors containing PAS domains have been assigned to the F6 system which would support the assignment of PSPTO\_2441 to this chemosensory system. The BACTH assays revealed interaction of PSPTO\_2441 with both CheA2 and CheA3. This analysis opens the door for more research on PSPTO\_2441 regarding its clustering in chemoreceptor arrays and its role in PsPto chemoperception.

Using cryo-electron tomography we have visualized two distinct chemoreceptor arrays in PsPto, which correspond to arrays of the F6 and F8 classes. Surprisingly, in the  $\Delta cheA2$  mutant background we still observe the presence of F6 arrays. A similar result has been observed in the case of *Azospirillum brasilense* mutant strains lacking the Che1 and Che4 clusters, corresponding to F5 and F7 systems (O'Neal et al., 2019). In this case, authors suggest the formation of mixed baseplates in the arrays, which would support the formation of the arrays in the absence of any of the CheA proteins (O'Neal et al., 2019). However, we cannot discard the possibility of arrays still forming because of the presence of CheW proteins. Actually, array formation in the absence of CheA has been experimentally shown to be possible in *Vibrio cholerae* (Yang et al., 2018). We determined that a strain lacking the *cheW* genes (PSPTO\_1985 and PSPTO\_1986) encoded in cluster I (F6 system) loses all swimming capacity (Figure S4), the same phenotype exhibited by a  $\Delta cheA2$  strain. Therefore, we can assure that PSPTO\_1985 and PSPTO\_1986 are needed to support swimming motility in PsPto. Further microscopy analyses in the  $\Delta cheW$ -F6 strain are needed to unveil these aspects. If this strain displayed no F6 arrays, we could therefore assure that these CheWs are specific for F6 array formation and no mixed baseplates occur in the formation of such arrays.

Our results confirm that the F6 system is required for chemotaxis and for plate swimming motility in PsPto. In contrast, the kinases from the F8 and ACF systems of PsPto do not seem to be involved in the regulation of chemotaxis in capillary assays, but they have a function in modulating motility in

plate swimming assays. These results are consistent with previous reports in other bacteria with more than one chemosensory system, showing the function of different chemotaxis pathways regulating distinct motility parameters. In *Rhizobium leguminosarum*, the Che1 pathway has been found to be the major chemotaxis cluster controlling motility pattern, while Che2 plays a minor role (Miller et al., 2007). In the case of *A. brasilense*, Che1 controls swimming speed and has only a minor role in chemotaxis, while the Che4 system controls cell tumbling, which is essential for chemotaxis (Mukherjee et al., 2016). Our results on the main role of CheA2 kinase in swimming motility and therefore of the F6 chemosensory system are in line with previous results from Clarke et al. (2016). However, we also found a role for the CheA1 and CheA3 kinases in the modulation of swimming motility that was not reported in this previous work. It is worth highlighting that the authors constructed two types of mutant strains, disruption and deletion mutants, and the described data is based on the disruption mutants' phenotypes. We have constructed clean deletion mutants and observed no defects in growth in M9 media supplemented with 10 mM citrate (Figure S5). Moreover, in *P. syringae* pv *tabaci*, a pathovar close to PsPto, the key role of the CheA2 kinase in the control of both chemotaxis and swimming motility, and a minor implication of the CheA1 kinase in these phenotypes had previously been shown (Ichinose et al., 2023). However, there is no data on the putative function of the kinase from the ACF system in this pathovar. Notably, no complementation was possible in any of the previous works showing phenotypes for CheA1 homologues. This could be due to the nature of a tight regulation of kinases functions.

Our observations reveal how CheY2 is the true chemotaxis response regulator and works together with CheA2 to ensure chemotaxis and swimming. However, while CheY1 does not seem to have a role in motility, the CheA1 kinase contributes to the control of this trait. This reveals how CheA1 and CheY1 work independently regarding motility. In bacteria containing more than one CheY homologue (Gao et al., 2021; Porter et al., 2011; Sourjik & Schmitt, 1996), it is common that one homologue is essential for the chemotaxis response while the remaining one works as a phosphate sink. This is the case for *Sinorhizobium meliloti* and *Azorhizobium caulinodans*. It is consistent that a mutation in the essential response regulator abolishes swimming behavior, while a mutation in the phosphate sink response regulator decreases swimming performance (Kennedy et al., 2022; Sourjik & Schmitt, 1996). However, this does not seem to be the case in PsPto, as a  $\Delta cheY1$  mutant does not show any defect in swimming motility. The dual role of two CheY homologues was also assessed in *Agrobacterium fabrum* (Gao et al., 2021). Here, CheY1 and CheY2 from *A. fabrum* were discovered to both contribute to chemotaxis by binding FlhM with different affinities, which culminated in different rates of swimming reversals, but both were shown to interact with the same CheA (Gao et al., 2021). In PsPto, we have shown the interaction between CheY2 and FlhM, although interaction between CheY1 and FlhM could not be detected. This could explain the absence of a swimming phenotype in the  $\Delta cheY1$  mutant.

Biofilm has been primarily described to be a feature under the control of ACF systems. However, several reports over the last years have shown how F systems also contribute to the regulation of biofilm formation (Berne & Brun, 2019; Huang et al., 2019; Liu et al., 2018a; O’Neal et al., 2019). Indeed, we have observed an increased biofilm formation in  $\Delta cheA1$  and  $\Delta cheA2$  mutant strains. The highest attachment capacity (which is the first step of biofilm formation) observed in the  $\Delta cheA2$  mutant aligns with the phenotype observed in a *cheA* mutant strain of *E. coli* (Suchanek et al., 2020). Moreover, these observations are in line with the findings in *Pseudomonas aeruginosa* PAO1 where the chemotaxis CheA kinase has been described to interact with the biofilm dispersal protein DipA phosphodiesterase, involved in the control of c-di-GMP homeostasis (Xin et al., 2019).

Twitching motility is performed via the type IV pili and serves as a surface exploration mechanism and contact with surfaces (Luo et al., 2015). Type IV pili and flagella have been found to be necessary for biofilm formation (O’Toole & Kolter, 1998). Moreover, Häußler et al. (2003) showed how *P. aeruginosa* isolates that displayed high autoaggregation were hyperpiliated. The higher presence of pili related proteins would explain the greater twitching capacity of the  $\Delta cheA$  mutants, as well as their flocculation behavior.

Surprisingly, in terms of biofilm related traits, mutants in the CheY response regulators displayed opposite phenotypes than those of the mutants in kinases encoded in the same clusters. This suggests that CheYs work independently from their cognate kinases to promote biofilm formation. A similar situation was reported in *Shewanella oneidensis*, where it was shown that phosphorylated CheY3 is essential for biofilm formation and that its phosphorylation comes from a different kinase than its cognate kinase CheA3 (Boyeldieu et al., 2020). They also showed that CheY3 interacts with Mxd proteins and the diguanylate cyclase PdgB. Mxd proteins are c-di-GMP effectors that belong to the glycosyl transferase family and are involved in polysaccharide production (Gambari et al., 2019). Other signaling crosstalk has been proposed for *Agrobacterium tumefaciens*, where its CheY2 response regulator would be additionally phosphorylated by a different kinase than its own cognate kinase to modulate flagellar motility (Guo et al., 2017). Phenotypes obtained for the  $\Delta cheY$  mutants correlate with low levels of intracellular c-di-GMP, suggesting their role in the control of the level of this second messenger. Low levels of c-di-GMP correlate with decreased biofilm and high motility, as the expression of flagellar genes and biofilm related genes is inversely regulated (Baraquet et al., 2012; Hickman & Harwood, 2008). It is important to note that this finding does not contradict the impaired motility of the  $\Delta cheY2$  mutant. It would be expected that a strain with low levels of c-di-GMP had an increased motility. However, the lack of CheY2 impedes the transduction of signaling to the flagellar motor at the level of the interaction with FlhM, resulting in a non-chemotactic strain regardless of its c-di-GMP levels.

Phenotypes of the  $\Delta wspR$  mutant strain correlate with those of the  $\Delta cheA3$  mutant strain. Both strains showed low swimming motility and enhanced biofilm formation, correlating with high c-di-GMP levels, which suggest a tandem activity of CheA3 kinase - WspR response regulator in the control of these traits. Extensive work has been done in *P. aeruginosa*, showing the WspR diguanylate cyclase activity as a response to the stimulation of the WspA chemoreceptor, resulting in increased c-di-GMP levels and biofilm formation (Güvener & Harwood, 2007; Hickman et al., 2005; O'Connor et al., 2012; O'Neal et al., 2022). Our work about the ACF system does not align with most of the literature available on the topic. Armbruster et al. (2019) reported that a deletion of *wspR* in *P. aeruginosa* resulted in similar amounts of biofilm relative to WT at later stages (24h), suggesting that any of the many other c-di-GMP cyclases present in this bacterium may ultimately compensate for c-di-GMP production in the absence of WspR. In the PsPto genome, there are 56 genes encoding putative c-di-GMP cyclases (He & Chou, 2016). These activities could account for a compensation activity in the  $\Delta wspR$  mutant background. Consequently, they might also contribute to compensatory mechanisms in the  $\Delta cheA3$  mutant, where the signaling pathway is impaired. However, other regulatory mechanisms leading to the modulation of the levels of c-di-GMP could be taking place.

BACTH analysis revealed unexpected interactions of WspR with both CheA1 and CheA2, which could explain the enhanced biofilm related phenotypes observed in the  $\Delta cheA1$  and  $\Delta cheA2$  mutants. As in the case of the signaling through CheA3, if these two kinases also participate in keeping the homeostasis of c-di-GMP through WspR, their mutation could induce a compensatory effect. However, we cannot discard interactions between CheAs and other biofilm regulators. Previous data on *Comamonas testosteroni* also reveals the interaction of the chemotaxis CheA kinase with two different response regulators, the chemotaxis response regulator CheY and the biofilm response regulator FlmD (Huang et al., 2019). Surprisingly, we were unable to detect the interaction between the kinase CheA1 and the response regulator CheY1 in our assays. This fact, together with the independence in the control of phenotypes observed in mutant strains in these two proteins, suggest that they do not really collaborate in the same signaling pathway (Figure 26).

Our results showed that both the kinases and the response regulators belonging to F6, F8, and ACF pathways are needed for full virulence in tomato plants. All chemosensory systems studied here are crucial in the stage involving an active entry process, as syringe infiltration assays restored *in planta* bacteria population of mutant strains. However, the ACF system seems to have an additional role involving symptom development, as leaves inoculated with mutants in the kinase and response regulator from this system displayed reduced necrotic areas when compared to the WT. All mutant strains except  $\Delta cheY1$  are affected in their swimming abilities, which explains the defect in virulence as it is well known that PsPto requires an active flagellar motility to incite infection (Hattermann, 1989).  $\Delta cheY1$  did not show a defect in motility but a phenotype in biofilm formation. Since PsPto is a

weak epiphyte, we could hypothesize that the epiphytic fitness of this strain is further compromised, rendering a lower infection ability.

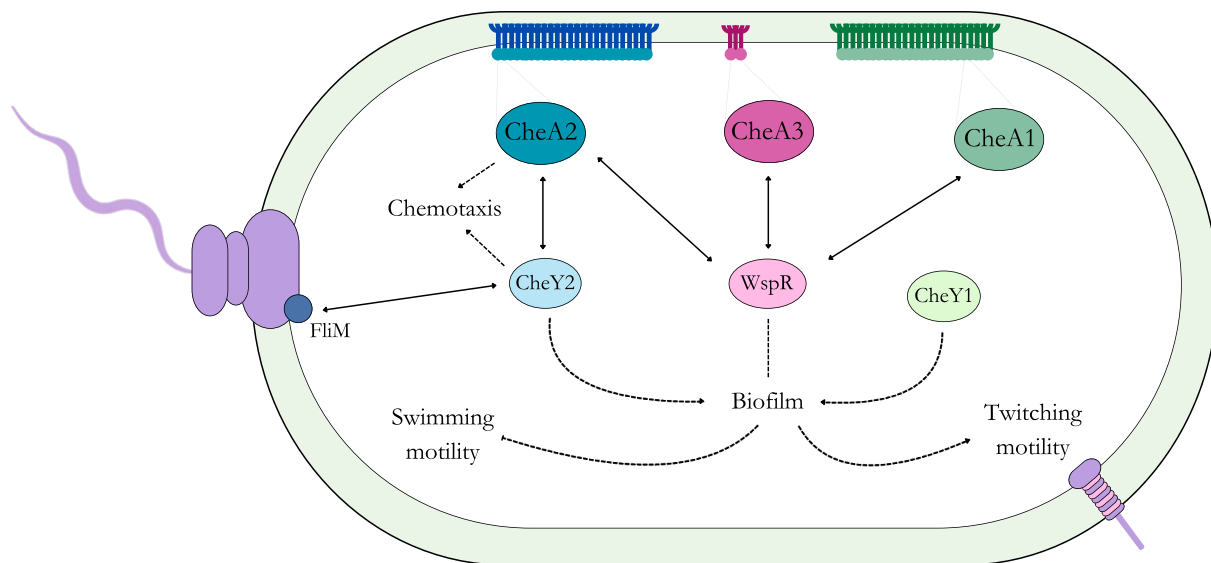


**Figure 26. Proposed model of signal transduction and crosstalk among chemosensory systems in PsPto.** Doubled-headed arrows indicate protein interaction. Solid lines indicate regulatory functions: arrow ends indicate promotion. Dashed lines indicate regulatory functions through unknown elements: arrow ends indicate promotion, dash ends indicate suppression, and no ends indicate unknown regulatory effects.

Taking all the results obtained, we proposed a model of signal transduction and crosstalk among chemosensory systems in PsPto (Figure 26). Chemoperception would signal through the response regulator CheY2 to modulate flagellar rotation through its interaction with FliM. Furthermore, CheA1, CheA2, and CheA3 would also interact with WspR, regulating biofilm formation, and both swimming and twitching motility. Since biofilm formation and flagellar motility are inversely regulated processes (Baraquet et al., 2012; Hickman & Harwood, 2008), CheA1, CheA3, and WspR functions could modulate both twitching and swimming motility through indirect intermediates, such as c-di-GMP levels (Aragón et al., 2015; Pérez-Mendoza et al., 2014). Our results also indicate how CheY1 and CheY2 response regulators do not align with their cognate kinases to regulate biofilm formation, where they act as biofilm promoters. CheY2 function would also promote twitching motility, again, probably through an indirect effect linked to biofilm regulation. These data suggest that both kinases and response regulators of PsPto chemosensory systems interact with additional partners to control key traits involved in virulence. Further analyses are needed to unveil the complex regulation network associated with chemosensory functions in this bacterium.

Overall, we show for the first time the visualization of the F6 and F8 chemosensory arrays of PsPto. Most of the chemoreceptors are assigned to the F6 system, which supports the chance for this visualization. The visualization of the F8 chemosensory system suggests that the only chemoreceptor protein known to associate with it (PSPTO\_0912) must have quite a relevant role in PsPto. Moreover,

our results unveil the crosstalk between the F6, F8, and the ACF systems at the level of the interaction between the CheA1 and CheA2 kinases with the response regulator WspR, pointing to this response regulator as a central sink of the chemosensory functions in PsPto for the control of virulence and related traits. This work shows novel results that contribute to the knowledge on chemosensory systems and their role in functions alternative to chemotaxis.



## **2. CHAPTER II**

***Pseudomonas syringae* pv. tomato infection of tomato plants is mediated by GABA and L-Pro chemoperception**



## 2.1. Introduction

The phyllosphere is a dynamic habitat where environmental conditions and nutrient availability vary spatially and temporally (Carvalho & Castillo, 2018; Sivakumar et al., 2020; Vorholt, 2012). This generates a selective environment for bacteria that can cope with adverse environmental conditions and can localize nutrient sources. For many bacterial pathogens, the phyllosphere provides nutrients that are released from the plant interior through leaching and guttation but also through wounds in damaged leaves or stomata (Ryffel et al., 2016; Vacher et al., 2016). As for many other bacterial pathogens, stomata and wounds are the main entry points for *Pseudomonas syringae* pv. tomato DC3000, where chemotaxis plays an active role in their detection.

As it has been previously mentioned, chemotaxis is crucial for detecting entry points like wounds or stomata and trigger the infection process. The abundance of chemoreceptor and chemotaxis genes in plant-associated bacteria has also been discussed earlier. More specifically, phytopathogenic bacteria harbor on average 27 chemoreceptor genes (Lacal et al., 2010), a number that is well above the bacterial average of 13 (Sanchis et al., 2021).

Many plant molecules have been found to be chemoattractants for plant pathogenic bacteria. These include amino acids, sugars, and organic acids present in the apoplast, or phenolic compounds released from wounds (Antúñez-Lamas et al., 2009; Ashby et al., 1988; Brewster et al., 2016; Hida et al., 2015, 2017, 2019; Kamoun & Kado, 1990; McKellar et al., 2015; Parke et al., 1987; Tumewu et al., 2020). However, there is scarce information about the signals that govern the switch from the epiphytic to the pathogenic stage in the phyllosphere.

Previous studies have determined that PsPto can perform chemotaxis towards amino acids, sugars, organic acids, and amides (Cerna-Vargas et al., 2019; Cuppels, 1988; Kim et al., 2007). However, despite the importance of chemotaxis and motility for PsPto virulence, only one of its 49 chemoreceptors has been previously characterized (Cerna-Vargas et al., 2019). As mentioned before, the chemoreceptor PsPto-PscA binds and mediates chemotaxis towards D-Asp, L-Asp, and L-Glu; and it was found to be required for full virulence in tomato plants (Cerna-Vargas et al., 2019). These results indicate the importance of amino acid perception in the plant infection process. Amino acids are abundant in the apoplastic tissue, providing a rich environment for plant pathogens. However, amino acids have been shown to be involved in plant defense (Yang et al., 2020), and their distribution varies depending on the physiological state of the plant and in response to abiotic and biotic stresses, including bacterial infection (Farvardin et al., 2020; O’Leary et al., 2016). This suggests that phytopathogens use amino acids not only as nutrients but also as signals to mediate plant interaction.

Gamma-amino-butyric acid (GABA) is a nonproteinogenic amino acid present in the apoplast of plants. Interestingly, GABA concentration increases significantly after PsPto infection (Ward et al.,

2010). Moreover, GABA has been observed to downregulate the expression of T3SS-related genes at elevated concentrations (Park et al., 2010). However, this regulation only occurs once infection has initiated (McCraw et al., 2016) and it has been speculated that it may coincide with the enhanced expression of genes involved in GABA catabolism, as observed in apoplastic populations of *P. syringae* 48 h after infection (Yu et al., 2013). These observations indicate that GABA acts not only as a nutrient of PsPto, but also as a signal to regulate its virulence inside the apoplast.

Several chemoreceptors from *Pseudomonas* species have been described to perceive GABA. The *Pseudomonas putida* McpG chemoreceptor specifically recognizes GABA (Reyes-Darias et al., 2015), while PctC of *Pseudomonas aeruginosa* perceives preferentially GABA but also binds L-Pro and L-His with lower affinity (Rico-Jiménez et al., 2013). In the phytopathogen *P. syringae* pv. tabaci the McpG chemoreceptor is implicated in GABA perception (Tumewu et al., 2020), while in *P. syringae* pv. actinidiae (Psa) the PscC chemoreceptor binds GABA and L-Pro (Ehrhardt et al., 2021; McKellar et al., 2015). The LBD of the PsPto chemoreceptor PSPTO\_2448 shares 97.7% sequence identity with the LBD of the PscC chemoreceptor of Psa. Interestingly, in addition to GABA, accumulation of proline has also been observed in *Arabidopsis* plants after PsPto infection (Gupta et al., 2020). It has been reported that this amino acid also plays a key role in plant defense against abiotic and biotic stresses (Verslues & Sharma, 2010).

PsPto-PscC (PSPTO\_2448) is a class Ia chemoreceptor belonging to cluster II, which contains a dCache\_1 LBD domain. This domain contains an amino acid recognition motif conserved in bacteria, archaea, and eukaryotes (Gumerov et al., 2022). Considering its conservation and the important role of amino acids as signals for both plants and PsPto, we investigated signal recognition by PsPto-PscC and its role during plant interaction.

## 2.2. Results

### 2.2.1. PsPto-PscC binds GABA and L-Pro

To identify the ligands recognized by PsPto-PscC, we purified its LBD by affinity chromatography and submitted the purified protein to thermal shift assays. This method records changes in the thermal stability of proteins caused by ligand binding (Martín-Mora et al., 2018). Increases in the midpoint of protein unfolding transition ( $T_m$ ) greater than 2°C are considered significant and indicative of binding. Significant increases of the recombinant protein  $T_m$  were observed in the presence of GABA and L-Pro in comparison to the ligand-free recombinant protein (Figure 27A). The PsPto-PscC-LBD recombinant protein was also tested against the PM3B phenotype compound array, which contains

amino acids and many other nitrogen sources. However, no significant increases in  $T_m$  were found for any compound present in the array (Figure S6). Isothermal titration calorimetry assays confirmed these results as the titration of PsPto-PscC LBD with GABA and L-Pro revealed binding with affinities of  $1.87 \pm 0.3 \mu\text{M}$  and  $4.52 \pm 0.4 \mu\text{M}$ , respectively (Figure 27B). Considering that the *P. aeruginosa* PctC chemoreceptor binds L-His in addition to GABA and L-Pro, we also performed microcalorimetric titrations with L-His. However, no binding heats were detected (data not shown). These results indicate that PsPto-PscC LBD binds specifically GABA and L-Pro, as observed for the Psa homologue PscC (McKellar et al., 2015).

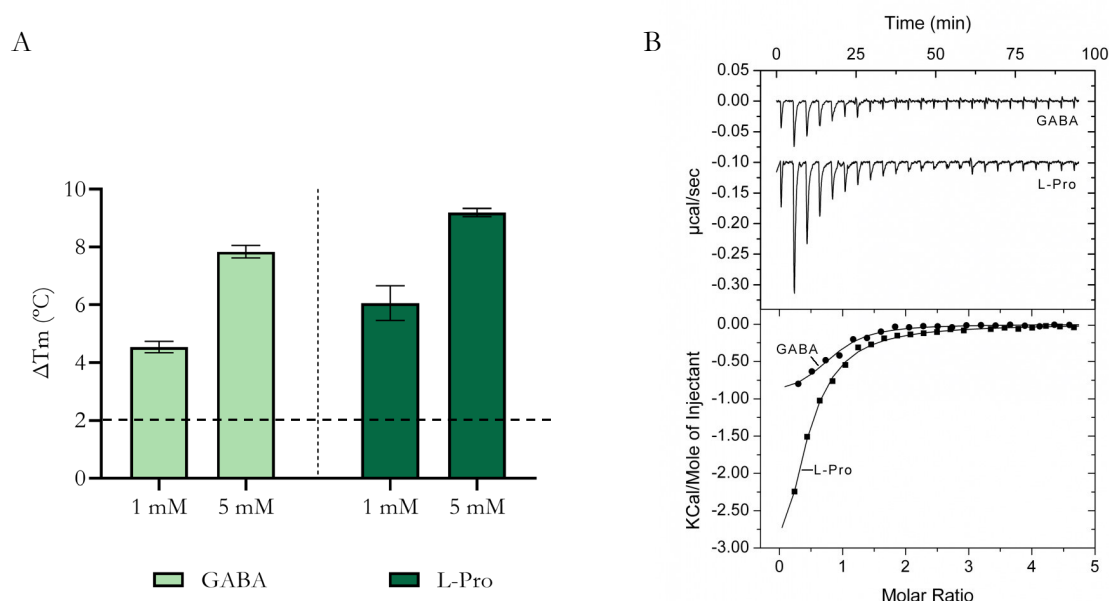


**Figure 27. The PsPto-PscC chemoreceptor binds specifically GABA and L-Pro.** (A) Differential scanning fluorimetry-based ligand screening of PsPto-PscC-LBD. Shown are means and standard errors of changes in the midpoint of the protein unfolding transition ( $T_m$ ) caused by the addition of 1 mM and 5 mM GABA or L-Pro with respect to the  $T_m$  of the ligand-free protein from three independent assays. The black dashed line highlights the threshold of 2°C, indicative of binding. (B) Microcalorimetric studies showing the binding of GABA and L-Pro to PsPto-PscC-LBD. Upper panel shows titration raw data for the injection of 4.2–8.6  $\mu\text{l}$  of 500  $\mu\text{M}$  ligand solutions into 13  $\mu\text{M}$  PsPto-PscC-LBD. Lower panel shows integrated, dilution heat-corrected, and concentration-normalized peak areas fitted with the one-binding-site model of ORIGIN.

### 2.2.2. PsPto-PscC mediates chemotaxis to GABA and L-Pro in PsPto

To evaluate the chemotactic response of PsPto to GABA and L-Pro, we performed quantitative capillary chemotaxis assays with the WT strain. Results obtained showed how GABA and L-Pro are chemoattractants for PsPto, with a maximum chemotactic response at 1 mM (Figure 28). To determine whether the chemotactic response observed was mediated through the PsPto-PscC chemoreceptor, we generated a defective PsPto mutant the *PsPto-PscC* gene. We performed quantitative capillary chemotaxis assays with the *pscC* mutant, and a significant reduction of chemotaxis was observed for

both GABA and L-Pro at 1 mM concentrations. Since no complete loss of chemotaxis was observed for any of the compounds tested, other chemoreceptors with overlapping ligand specificities must be present in PsPto. To verify that the ligand specificity found in ligand-binding experiments translates into a ligand specificity at the chemotactic level, we conducted quantitative capillary chemotaxis assays towards other amino acids (L-Asp, L-Glu, and L-Asn) with the WT and the *pscC* mutant strain. We did not detect any chemotaxis defect in the mutant strain compared to the WT strain, which corroborates the specificity of the PsPto-PscC chemoreceptor in mediating chemotactic responses to GABA and L-Pro (Figure S7). Complementation of the *pscC* mutant with the PsPto-PscC chemoreceptor expressed in trans resulted not only in full recovery of the chemotactic ability towards GABA and L-Pro, but in an increased chemoattraction in comparison to the WT strain (Figure S8). Expression in trans from multicopy plasmids enhances cellular chemoreceptor levels and previous studies following a similar approach have also reported an increase in the magnitude of the chemotactic response (Hida et al., 2020; Rico-Jiménez et al., 2022).

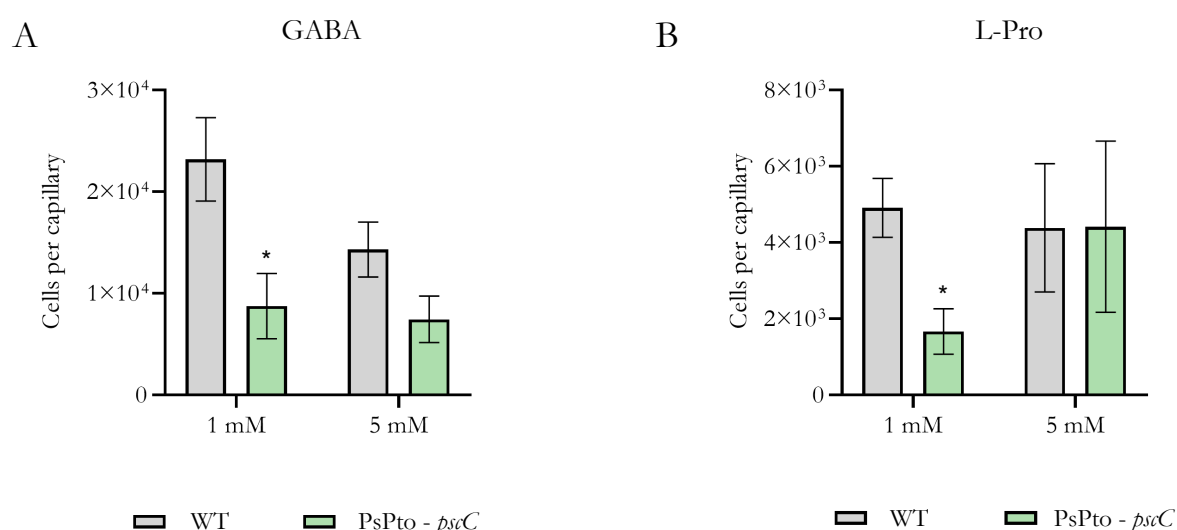


**Figure 28. Chemotaxis capillary assays of WT and the PsPto-*pscC* mutant strains towards GABA (A) and L-Pro (B).** The data have been corrected with the number of cells that swam into buffer-containing capillaries. Shown are means and standard errors from three independent experiments with three technical replicates each. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ).

### 2.2.3. GABA perception by the PsPto-PscC chemoreceptor regulates the expression of genes involved in GABA catabolism

In *P. syringae* pv. phaseolicola NPS3121 and *P. syringae* pv. *syringae* B728a the expression of genes involved in GABA catabolism is upregulated in the apoplast (Hernández-Morales et al., 2009; Yu et al., 2013). GABA is the most abundant amino acid in the tomato apoplast (Rico & Preston, 2008). Therefore, we wondered whether the expression of GABA catabolism genes was altered in response to the addition of GABA when growing in *hrp*-inducing minimal media (HIM), which mimics the

apoplast conditions. For these experiments, we chose to use GABA at a final concentration of 1 mM, a close value to that found in tomato apoplasts (Rico & Preston, 2008). We observed that the expression of *gabP*, which encodes a permease, was upregulated in comparison to the nontreated WT cells. *GabP* has been identified as the principal GABA transporter in PsPto (McCraw et al., 2016). Furthermore, we evaluated the expression of three genes encoding GABA transaminases, *gabT1*, *gabT2*, and *gabT3*, and the succinic semialdehyde dehydrogenase gene *gabD2*. Our results showed that the expression of *gabT1*, *gabT2*, and *gabD2* was upregulated after GABA addition compared to nontreated cells (Figure 29A). However, no differences were observed for the *gabT3* expression (Figure 29A). These results indicate how concentrations close to those found in tomato apoplast alter the expression of genes involved in GABA metabolism.

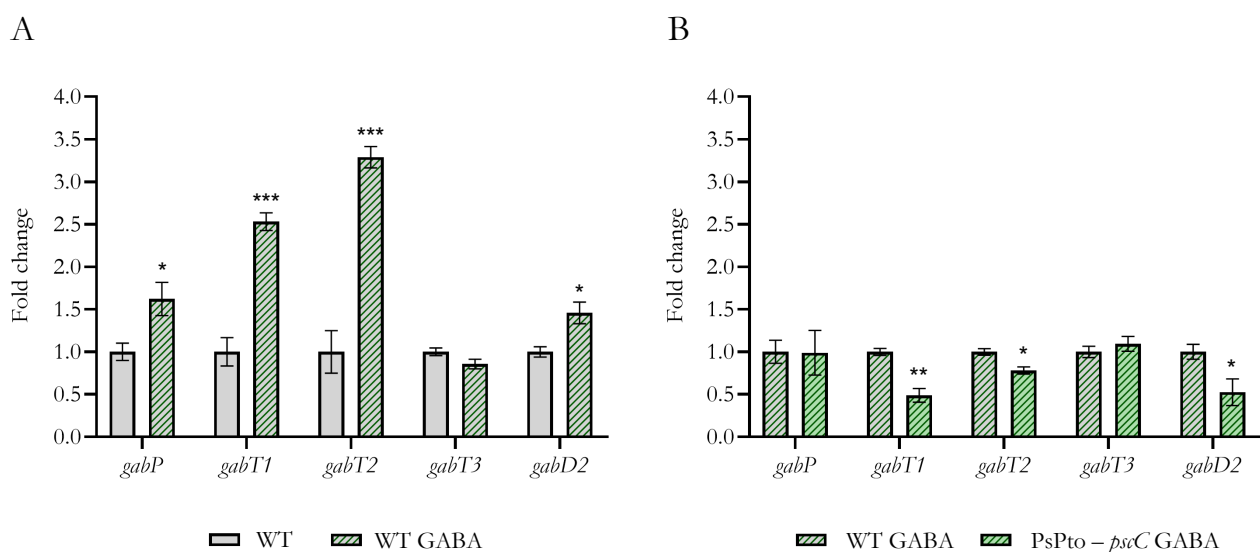


**Figure 29. Expression of GABA catabolic genes in response to GABA perception.** (A) Shown are means and standard errors of the relative expression changes, quantified by reverse transcription-quantitative PCR (RT-qPCR), in the WT strain in the presence (green-dashed grey bars) or absence (no-dashed grey bars) of 1 mM GABA. (B) Shown are means and standard errors of the relative expression changes, quantified by RT-qPCR, in the presence of 1 mM GABA in the PsPto-*pscC* mutant (green-dashed light green bars) with respect to the WT strain (green-dashed grey bars). Three independent experiments with three technical replicates each were performed. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.005$ ).

To assess whether this regulation is mediated through GABA perception by the PsPto-PscC chemoreceptor, we analyzed the expression of these genes in the *pscC* mutant strain, also after addition of 1 mM GABA. Results indicate that while the expression of *gabT1*, *gabT2*, and *gabD* was downregulated in the *pscC* mutant strain, the expression of *gabT3* and *gabP* showed no significant differences when compared to the WT strain (Figure 29B).

### 2.2.4. Expression of *hrpL* and *avrPto* is not altered in the *PsPto-pscC* mutant

GABA levels increase in the apoplast under biotic stress conditions (Farvardin et al., 2020; O’Leary et al., 2016; Park et al., 2010). This has been associated with a greater plant resistance against *PsPto* infection (Deng et al., 2020; Park et al., 2010). Furthermore, it has been reported that increased GABA levels are associated with a downregulation in the expression of T3SS-related genes (Deng et al., 2020; Park et al., 2010). Considering these previous findings, we assessed the expression of two important virulence related genes, *hrpL* and *avrPto*, in response to GABA perception. Consistent with Mccraw et al. (2016), we observed a downregulation in the expression of both genes compared to nontreated cells (Figure 30A). Next, we wondered if this regulation was controlled through GABA perception by the *PsPto*-PscC chemoreceptor. We assessed the expression of these two genes in both the WT and the *pscC* mutant strain after addition of 1 mM GABA. We did not observe any difference in the expression of the *hrpL* and *avrPto* in the *pscC* mutant strain in the conditions tested (Figure 30B).

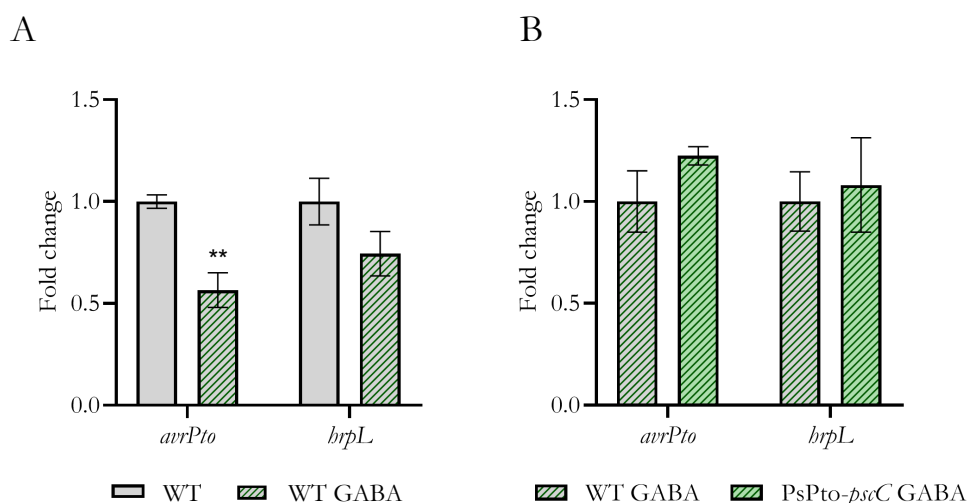


**Figure 30. Expression T3SS-related genes in response to GABA perception.** (A) Shown are means and standard errors of the relative expression changes, quantified by reverse transcription-quantitative PCR, in the WT strain in the presence (green-dashed grey bars) or absence (no-dashed grey bars) of 1 mM GABA. (B) Shown are means and standard errors of the relative expression changes, quantified by reverse transcription-quantitative PCR in the presence of 1 mM GABA in the *PsPto-pscC* mutant (green-dashed grey bars) with respect to the WT strain (green-dashed grey bars). Three independent experiments with three technical replicates each were performed. Values that are significantly different are indicated by asterisks (\*\* $p < 0.01$ ).

These results indicate that *PsPto*-PscC chemoreceptor does not mediate the expression of T3SS-related genes, a regulation that usually occurs in latter stages of infection.

### 2.2.5. PsPto-PscC function is required for full virulence in PsPto in tomato plants

Considering the reduced chemotaxis towards GABA and the altered gene expression exhibited by the *pscC* mutant strain, we evaluated its capacity to promote infection in tomato plants. To this end, we used two inoculation methods, namely the spray-inoculation approach, which mimics natural entry conditions that bacteria face in the phyllosphere, and the infiltration method, in which the pathogen is placed inside the plant. Spray-inoculation resulted in a significant reduction in the number of *pscC* mutant cells that entered the plant as compared to WT cells at 2 h postinoculation (hpi) (Figure 31A). This finding is supported by a reduced cell count in virulence assays as measured 6 days postinoculation (dpi) (Figure 31B). However, no significant differences between both strains were observed when plants were infiltrated (Figure 31B). The reduced entry and virulence of the *pscC* mutant strain were restored to WT levels by complementation with the *pscC* gene provided in trans (Figures 31A and Figure S9). These findings thus demonstrate that PsPto-PscC function is important to drive bacterial entry into the plant apoplast and that its mutation significantly alters infection severity in tomato plants.

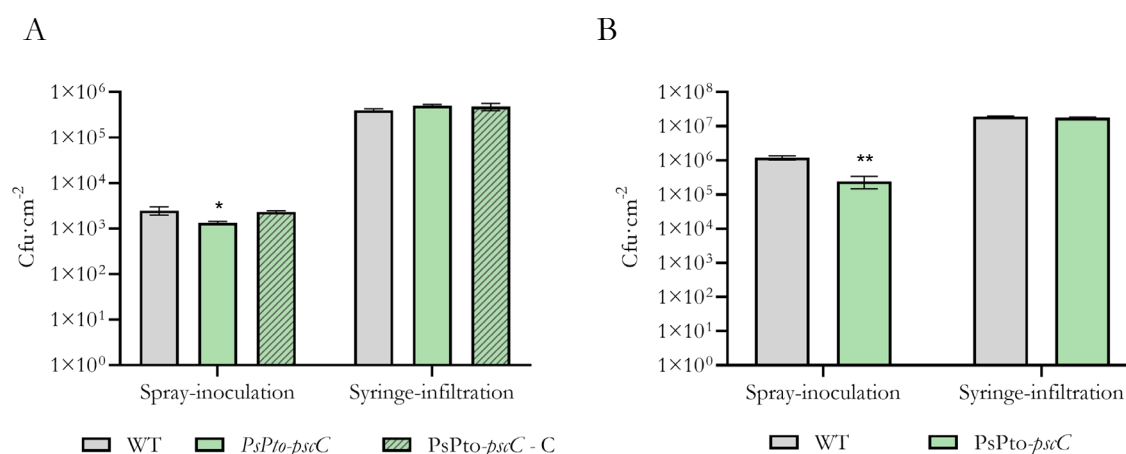


**Figure 31. Entry and virulence assays in tomato plants with two inoculation methods.** (A) Bacterial populations of the WT, the *PsPto-pscC*, and the *PsPto-pscC* - C complemented strains at 2 hpi after spray-inoculation ( $10^8$  cfu·ml<sup>-1</sup>) or syringe-infiltration ( $3 \times 10^4$  cfu·ml<sup>-1</sup>). (B) Bacterial populations of the WT and the *PsPto-pscC* mutant strains at 6 days postinoculation (dpi) after spray-inoculation ( $10^8$  cfu·ml<sup>-1</sup>) or at 3 dpi after syringe-infiltration ( $3 \times 10^4$  cfu·ml<sup>-1</sup>). Shown are means and standard errors from three independent experiments with three technical replicates each. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ ).

To get further insight into the role of GABA chemoperception during the infection process, we assessed virulence in *A. thaliana gad1/2* mutant plants, which present reduced GABA levels (Scholz et al., 2015b). Wild-type Col-0 and *gad1/2* *A. thaliana* plants were spray-inoculated with the WT and *pscC* mutant strains. Results did not show any difference in bacterial populations between the WT and

*pscC* mutant strains, independently of the *Arabidopsis* genotype assayed (Figure 32). Moreover, as previously reported by Xian et al. (2020), no differences were observed in infection severity between Col-0 and *gad1/2* *A. thaliana* plants (Figure 32).

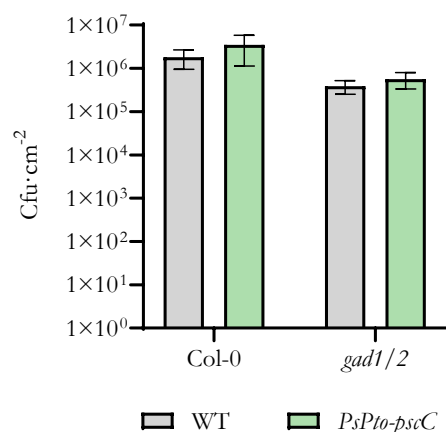


**Figure 32. Virulence assays in *Arabidopsis thaliana* plants.** Bacterial populations of the WT and *PsPto-pscC* mutant strains at 6 dpi ( $3 \times 10^8$  cfu·ml<sup>-1</sup>) of Col-0 or *gad1/2* plants. Shown are means and standard errors from three independent experiments with six technical replicates each.

## 2.3. Discussion

Apoplastic fluid has traditionally been considered as an important source of nutrients for bacterial pathogens, allowing their growth and development. Moreover, bacteria have evolved adaptations to exploit changes in plant metabolism induced after microbe perception (Leonard et al., 2017), allowing the use of apoplastic compounds as cues to regulate the interaction with the plant. Apoplastic compounds can also be considered as signals that could be recognized by the chemotaxis machinery of bacterial pathogens to enter the apoplast (Antúnez-Lamas et al., 2009; Matilla & Krell, 2018; Melotto et al., 2006).

Multiple lines of evidence indicate that the capacity to sense and respond to amino acids is crucial across many forms of life. (1) A recent study has identified an amino acid-specific sensor domain, dCache\_1AA, that is found throughout the Tree of Life in archaea, bacteria, and different eukaryotes (Gumerov et al., 2022). In bacteria, this domain is found in all major families of transmembrane receptors, including chemoreceptors, sensor kinases, guanylate/adenylate cyclases, cyclic-di-GMP phosphodiesterases or serine/threonine kinases and phosphatases. (2) In bacteria, multiple mechanisms have evolved to sense amino acids. Next to a direct recognition at different types of LBDs, namely dCache\_1AA, TarH, or DAHL (Matilla et al., 2022), there is evidence for transmembrane receptor stimulation by the binding of amino acid-loaded solute-binding proteins (Matilla et al., 2021). (3) The ligand profiles of many bacterial chemoreceptors have so far been determined (Matilla et al., 2022). However, the by far most abundant family is formed by amino acid-responsive chemoreceptors that show a wide phylogenetic spread (Matilla et al., 2022). In the framework of the general importance of amino acid sensing in life, this study illustrates the central role of sensing these ligands in the context

of plant infection. As for phytopathogens, amino acid composition of the apoplast and root exudates were found to change significantly after bacterial infection (O’Leary et al., 2016; Yuan et al., 2018). Taking this data together, amino acids may represent key players in the signaling process between plants and bacteria during infection.

Only a limited number of amino acid-specific chemoreceptors have been functionally characterized in phytopathogenic bacteria (Cerna-Vargas et al., 2019; McKellar et al., 2015; Rico-Jiménez et al., 2013; Tumewu et al., 2020; Tumewu et al., 2021). In this work we have identified the ligands of the PsPto-PscC chemoreceptor of PsPto. We found that PsPto-PscC binds specifically to GABA and L-Pro, consistent with what was observed for the PscC homologue in *Psa* (McKellar et al., 2015). However, the characterization of the role of *Psa*-PscC during the interaction with the plant has not been addressed in *Psa*.

In plants, GABA and proline are involved in carbon/nitrogen metabolism and in the regulation of different aspects of plant physiology (Tarkowski et al., 2020). However, these amino acids also play a key role as signaling molecules during the plant response to different stresses, including pathogen infection (Seifikalhor et al., 2019; Tarkowski et al., 2020). Under stress conditions, GABA levels increase in the apoplast due to the activation of the glutamate decarboxylase enzyme GAD through  $\text{Ca}^{2+}$  signaling (Tarkowski et al., 2020; Ward et al., 2010). Similarly, L-Pro levels increase by the activation of the enzyme pyrroline-5-carboxylate synthase (P5CS), involved in L-Pro synthesis from L-Glu (Mattioli et al., 2009). Moreover, proline can be converted into GABA through a nonenzymatic reaction under stress conditions (Signorelli et al., 2015), and in the case of *Arabidopsis* plants, some GABA transporters are also able to transport proline (Breitkreuz et al., 1999). PsPto-PscC LBD bound GABA and L-Pro with high affinity as evidenced by dissociation constants ( $K_D$ ) values in the lower micromolar range, which are comparable to those obtained for PscC from *Psa* (Ehrhardt et al., 2021) and PctC from *P. aeruginosa* (Rico-Jiménez et al., 2013). For PctA and PctB, two other amino acid-responsive dCache-containing chemoreceptors in *P. aeruginosa*, a correlation has been established between high ligand affinity and highly sensitive responses (Reyes-Darias et al., 2015). The capacity to mediate responses to low ligand concentrations may be a feature common to the GABA- and L-Pro-responsive chemoreceptors in pseudomonads.

The PsPto-PscC mutant showed some residual responses to both of its ligands, indicative of the existence of additional chemoreceptors that respond to these amino acids, a finding that may not surprise considering that PsPto encodes 49 chemoreceptors. Previous studies showed that GABA and L-Pro are not preferred nutrients for PsPto and that they are only used when the preferential nutrients have been consumed (McCraw et al., 2016). The described chemoattraction to these compounds, regardless of their nutritional value, may indicate that PsPto uses the perception of these amino acids as a cue to localize the apoplast and initiate infection. Similarly, the use of nonpreferred amino acids

as signals to locate nutrients and start the interaction with the plant has been previously proposed for *Bacillus subtilis* (Yang et al., 2015a).

The PsPto genome encodes three GABA transaminases and three succinate-semialdehyde dehydrogenases (Park et al., 2010). However, PsPto is unable to synthesize GABA directly from glutamate due to the lack of a GadB enzyme (McCraw et al., 2016). Therefore, it is tempting to speculate that this bacterium obtains GABA from the apoplast of plants, where it is more abundant. The unusually high number of GABA catabolic enzymes in the PsPto genome may be related to a particular importance of GABA metabolism during the interaction with the plant host.

In a previous transcriptomic analysis carried out in *P. syringae* pv. *syringae* B728a, it was observed that the expression of *gabP* and GABA catabolic genes was upregulated in planta and that their expression was increased in cells growing in the apoplast as compared to epiphytic populations (Yu et al., 2013). We show here that the addition of GABA to PsPto growing in HIM increased *gabP* expression. GabP represents the main mechanism of GABA uptake in PsPto (McCraw et al., 2016). We also observed up-regulation of the encoding succinate-semialdehyde dehydrogenase (*gabD2*) and transaminases (*gabT1* and *gabT2*), but not of *gabT3*. It has been suggested that the *gabT3* gene, found in a different location of the genome, may not be a GABA transaminase (Park et al., 2010). Interestingly, we observed that in the presence of GABA, the expression of *gabD2*, *gabT1*, and *gabT2*, but not that of *gabP*, was down-regulated in the *PsPto-pscC* mutant strain, which suggests that the perception of GABA through this chemoreceptor regulates the expression of GABA catabolism genes.

The expression of T3SS genes, the main virulence factor in PsPto, is induced in the presence of plant-derived signals (Anderson et al., 2014; Tang et al., 2006). A recent work has reported that the two-component system AauS–AauR in PsPto directly connects the perception of host-derived amino acids (aspartate and glutamate) to the up-regulation of T3SS-encoding genes (Yan et al., 2020). Interestingly, the presence of increased levels of GABA down-regulates the expression of T3SS-related genes in PsPto (Park et al., 2010). Accordingly, we found that the expression of *avrPto* and *hrpL* genes was down-regulated in the WT strain in the presence of 1 mM GABA and that this regulation was not mediated by PsPto-PscC. In PsPto, the repression of T3SS-related genes is mediated by GABA uptake through the GabP permease (McCraw et al., 2016) and the expression of *gabP* was similar in the WT and the *PsPto-pscC* mutant strain. Interestingly, while the expression of the *B. subtilis* GABA catabolic genes *gabT* and *gabD* is under the control of the regulatory protein GabR, the expression of *gabP* is not regulated by this protein (Belitsky & Sonenshein, 2002).

We propose that PsPto-PscC has a role as GABA chemoreceptor in the early stages of plant interaction, namely the epiphytic phase, to locate entry sites and navigate towards them, ensuring apoplast invasion. Consistently, we show here that the reduced chemotactic response towards GABA and L-Pro observed in the mutant strain caused an impaired leaf entry and reduced virulence in tomato

plants. We did not observe differences either in entry or in virulence between the WT and the mutant strain in *A. thaliana* plants. PsPto infected *A. thaliana* WT and *gad1/2* mutant plants, which produce lower GABA levels, in a similar manner. This is consistent with the proposition that PsPto virulence in *Arabidopsis* is not influenced by GABA (Xian et al., 2020), as it is the case in tomato plants. Under nonstress conditions, GABA levels are lower in *Arabidopsis* than in tomato (Deng et al., 2020; Rico & Preston, 2008). Although GABA levels can suffer a threefold increase during biotic stress in *Arabidopsis* (Ward et al., 2010), the concentration is still well below that found in the tomato apoplast, which is around 500  $\mu$ M (Rico & Preston, 2008) under nonstress conditions and can increase to 2.5 mM under biotic stress (Solomon & Oliver, 2002). These data suggest that the signals perceived by PsPto during infection of tomato and *Arabidopsis* are different.

Our data highlight the key role of GABA and L-Pro as plant-released chemoeffectors perceived by PsPto to drive entry into the tomato host plant. These results are in line with the suggested pivotal role of amino acid perception during bacterial plant infection. The differential role of this perception in *Arabidopsis* plants suggests that the specific profile of pathogen chemoperception functions is under evolutionary pressure of the presence of host-specific signals.



### **3. CHAPTER III**

**Energy taxis in *Dickeya dadantii* 3937 is governed by three PAS domain-containing chemoreceptors**



### 3.1. Introduction

Many phytopathogenic bacteria face an epiphytic stage in their infection process, where they need to cope with many environmental stresses. Bacteria can then enter the plant apoplast through natural openings like stomata or through wounds. As mentioned throughout this thesis, chemotaxis plays a crucial role in the initial stages of bacterial plant pathogenesis. Once inside the apoplast, bacteria face other stresses. In the case of *Dickeya dadantii* 3937, these are mainly acidic shock, microaerophilic conditions, low iron availability, and ROS and antimicrobial peptides produced by the plant host.

Chemotaxis has been classically defined as a response to external chemical inputs (Adler, 1969). However, these chemical cues can also come from the inside of the cell. Energy taxis is a form of chemotaxis which enables bacteria to perceive their inner metabolic state and move towards energetically advantageous niches (Schweinitzer & Josenhans, 2010). Stimuli is also perceived through chemoreceptors or MCPs, and the signal is transmitted through CheA and CheY to modulate flagellar rotation. Chemoreceptors known to mediate energy taxis display different LBD structures. As mentioned in earlier sections, the most common are those including PAS domains, and the best characterized energy taxis sensor containing a PAS domain is the Aer chemoreceptor from *E. coli*. PAS domains, as mentioned in previous sections, are proteins widely distributed in all kingdoms of life which can sense light, oxygen, or redox potential as input signals (Taylor, 2007). *E. coli* Aer contains a cytoplasmic PAS signaling domain which binds Flavin Adenine Dinucleotide (FAD), a transmembrane region and the conserved cytoplasmic HAMP domain followed by the signaling domain. The redox status of FAD would induce conformational changes in the PAS domain, transducing the signal to the Che proteins through the HAMP and kinase control domains, which ultimately control flagellar rotation (Alexandre et al., 2004; Sporer et al., 2017). Furthermore, it is thought that NADH dehydrogenase I from the electron transport chain (ETC) couples with Aer by generating reducing equivalents of low enough potential to donate electrons to the receptor, possibly through protein-protein interactions (Maschmann et al., 2022).

FAD is a major coenzyme for redox reactions, involved in several metabolic pathways. This flavin nucleotide can exist in five redox states, which are the flavin-N(5)-oxide oxidized form, neutral and anionic semiquinones, and neutral and anionic fully-reduced hydroquinones. FAD is converted between these states by accepting or donating electrons. FAD, in its fully oxidized form, or quinone form, accepts two electrons and two protons to become FADH<sub>2</sub> (hydroquinone form). The semiquinones (FADH<sup>•</sup>) can be formed by either reduction of FAD or oxidation of FADH<sub>2</sub> by accepting or donating one electron and one proton, respectively (Teufel et al., 2015). Redox changes in FAD reflect redox potential changes in the electron transport chain that result from an increase or decrease in oxygen concentration.

The presence of FAD is essential to the sensory function of the Aer-type of receptors, since proteins unable to bind this cofactor fail to mediate a response to changes in oxygen concentrations (Alexandre, 2010). Aer may detect aerotactic stimuli by using noncovalent FAD-binding to monitor cellular redox state. In this way, it functions as a conventional chemoreceptor by reversibly binding FAD, with differential affinity for its oxidized and reduced forms (Bibikov et al., 2000). PAS domain transduces FAD redox status to the highly conserved signaling domain in the C-terminus of the chemoreceptor, generating the on and off signals for energy taxis (Maschmann et al., 2022; Taylor et al., 1999). The signaling domain forms a complex with the CheA and CheW proteins and modulates locomotor behavior through the flagellar motor (Bibikov et al., 2000; Taylor et al., 1999). According to Repik et al. (2000), there are twelve residues involved in signal transduction in the Aer PAS domain (N34, G42, E47, R57, H58, D60, F66, D68, W79, N85, G90 and Y111). Moreover, the residue W70 has been described to participate in the interaction with FAD, being the only irreplaceable position for ligand binding (Xie et al., 2010).

It has been postulated that the Aer PAS domain interacts with the electron transport components. However, the reduction potential of the FAD cofactor in Aer seems not to be within a range that would permit electron exchange with ubiquinone  $\pm$  menaquinone via the electron transport system and it is not clear if other intermediate reductants are involved in the process (Maschmann et al., 2022). That is why it is thought that Aer might interact with the NADH dehydrogenase to obtain electrons. Maximal respiration rates (saturating electron donor and optimal electron acceptor) favor formation of the semiquinone form of ubiquinone (menaquinone). If the FAD cofactor of Aer is in equilibrium with the quinones of the respiratory system (even through an intermediate reductant), the semiquinone form of FAD ( $\text{FADH}\cdot$ ) would be predominant at maximal respiration. These steady-state conditions are associated with CCW rotation of the flagella and prompts the bacteria to move in runs. The fully oxidized quinone (FAD) or fully reduced hydroquinone ( $\text{FADH}_2$ ) generate a CW signal causing tumbles (Maschmann et al., 2022; Repik et al., 2000; Taylor, 2007) (Figure 33).

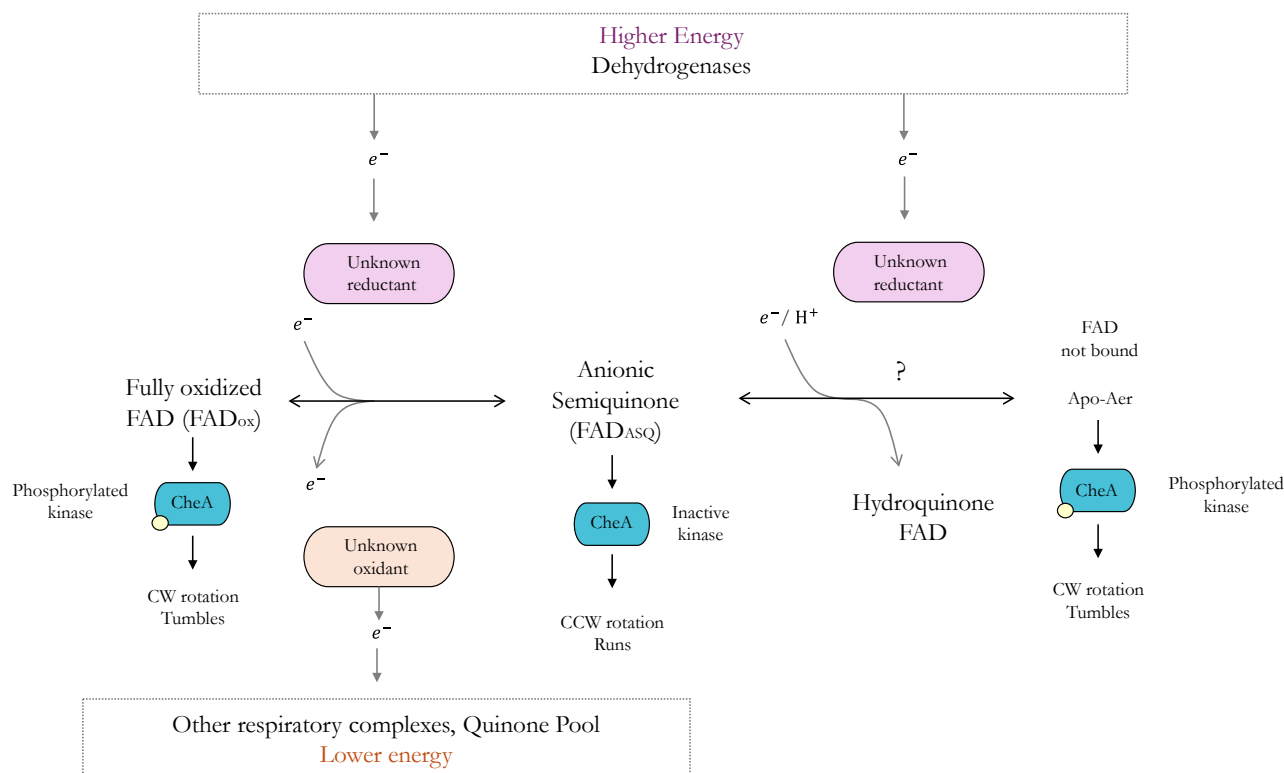


**Figure 33. Aer energy sensing model.** An unknown higher energy reductant reduces  $FAD_{ox}$  to  $FAD_{ASQ}$ , switching the Aer signaling state from phosphorylated CheA to unphosphorylated CheA. Aer- $FAD_{ASQ}$  then reduces an unknown oxidant, switching the signaling state back. Thus, Aer signaling events are determined by the availability of both higher energy reductant species and lower energy oxidant species, conferring sensitivity to the flow of reducing equivalents within respiratory chains at higher and lower energies, “upstream” and “downstream.” FAD, flavin adenine dinucleotide;  $FAD_{ASQ}$ , FAD anionic semiquinone;  $FAD_{ox}$ , oxidized FAD.

Several chemoreceptors structurally homologous to *E. coli* Aer have been studied at different levels in plant associated bacteria: *Ralstonia solanacearum* Aer1 and Aer2 (Jian & Caitilyn, 2006), *Azospirillum brasilense* AerC (Xie et al., 2010), *P. clororaphis* PCL1606 Aer1-1 and Aer1-2 (Arrebola & Cazorla, 2020), and *P. syringae* pv. tabaci AerB and AerC (Tumewu et al., 2022).

*D. dadantii* 3937 contains 47 chemoreceptors signaling through a single chemosensory system. Only four chemoreceptors contain a PAS domain as LBD, three of which are anchored to the membrane, and one is completely cytoplasmic. DDA3937\_RS12290, DDA3937\_RS17070, and DDA3937\_RS12165 are structurally homologous to *E. coli* Aer (Figure 34). In this work, we characterize these chemoreceptors in the context of energy taxis and bacterial pathogenesis.

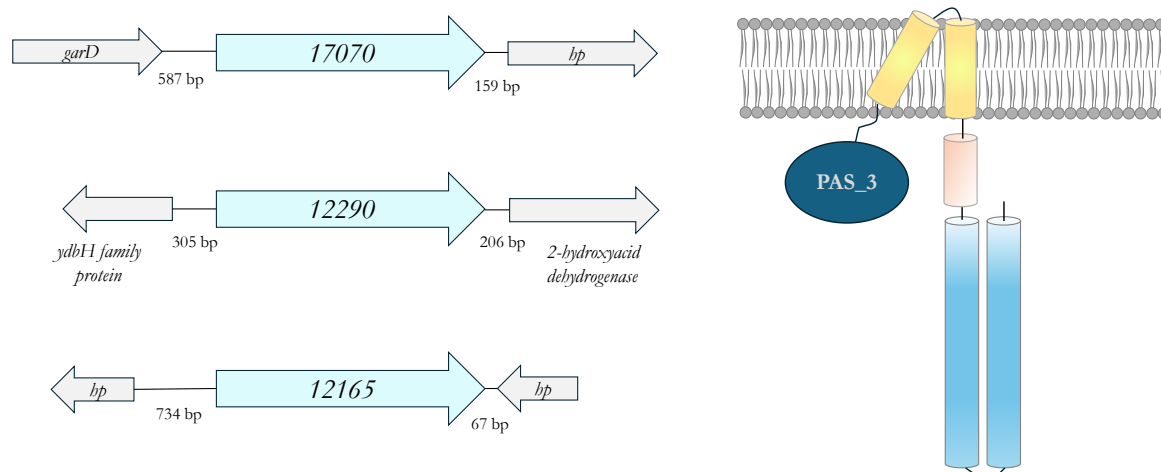


**Figure 34. Aer-like chemoreceptors of *Dickeya dadantii* 3937.** Schematic view of the genomic context of *DDA\_RS17070*, *DDA\_RS12290*, *DDA\_RS12165* and structure of a canonical Aer chemoreceptor.

## 3.2. Results

### 3.2.1. Mutation of Aer-like chemoreceptors affect growth on different substrates

We generated three single mutant strains for all Aer-like chemoreceptors (*17070*, *12290*, and *12165*), three combinatorial double mutants (*17070-12290*, *17070-12165*, *12165-12290*), and a triple mutant (*17070-12290-12165*). We characterized their ability to grow on diverse substrates: rich 1/2× KB medium and minimal media M63 supplemented with 30 mM succinate. Growth curves are shown in Figure 35.

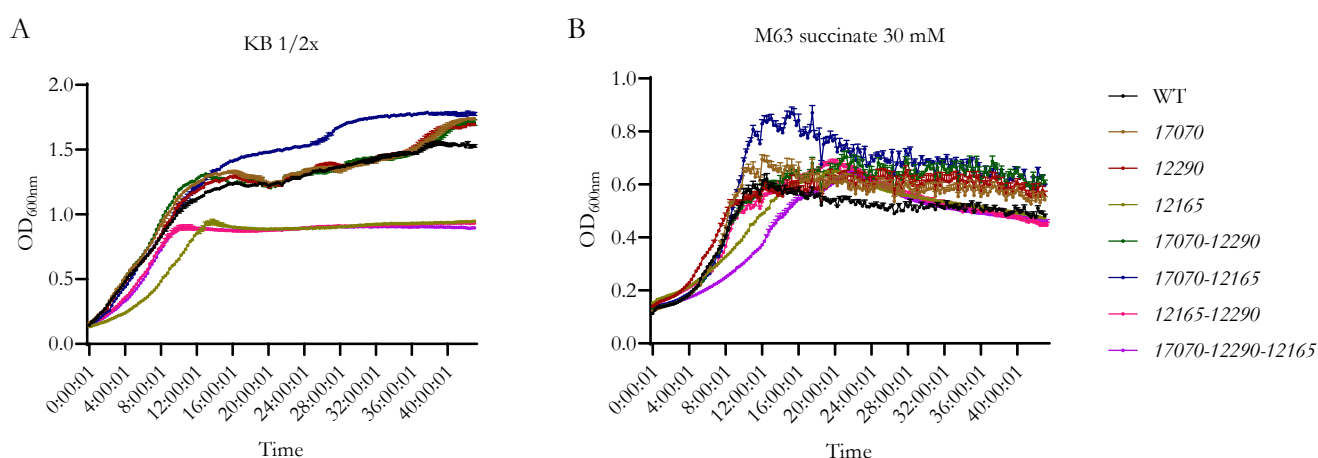


**Figure 35. Growth curves of mutant strains in KB and M63 medium supplemented with 30 mM succinate.** Measurements were taken every 15 minutes using Bioscreen C° Pro. Three independent experiments were performed with eight technical replicates each.

Most strains grew similarly to the WT strain in KB medium. However, the single *12165*, the double *12165-12290*, and the triple *17070-12290-12165* mutant displayed a slower growth rate than the WT strain.

We obtain similar results when we assay the growth capacity of *Aer*-like mutants in M63 minimal medium supplemented with succinate. However, in this case, the double mutant *12165-12290* displays a similar growth curve than the WT. Notably, the differences in growth rate displayed by the single *12165* and the triple *17070-12290-12165* mutant are more severe when succinate is used as a sole carbon source.

### 3.2.2. *Aer*-like chemoreceptors modulate swimming motility in Dd3937

As mentioned in earlier sections, energy taxis allows bacteria to modulate flagellar motility to move towards energetically advantageous niches (Schweinitzer & Josenhans, 2010). We assessed the ability of *Aer*-like mutant strains to perform swimming motility in both rich media and minimal media M63 supplemented with 30 mM succinate (Figure 36). We observed how all strains are capable of performing swimming motility in all media tested, which indicates that none of these chemoreceptors is essential for swimming motility. All mutant strains display a significantly smaller swimming diameter than the WT in rich media. However, only the *12165*, *17070-12165*, *12165-12290*, and *17070-12290-12165* displayed smaller swimming diameters than the WT in M63 medium supplemented with 30 mM succinate.

The respiratory activity of motile cells growing on agar depletes the oxygen supply and creates an oxygen gradient that forces bacteria to swim outwards the inoculation site. Moreover, a characteristic band of cells congregated at the leading edge of the oxygen gradient appears when their ability to perceive oxygen is intact, as previously described by Bibikov et al. (2000). While the WT strain, *17070*, *12165*, and *17070-12165* present this characteristic band of cell accumulation at the edge of the colony, mutant strains *12290*, *17070-12290*, *12165-12290*, and *17070-12290-12165* lost this trait (Figure 36, white arrows).

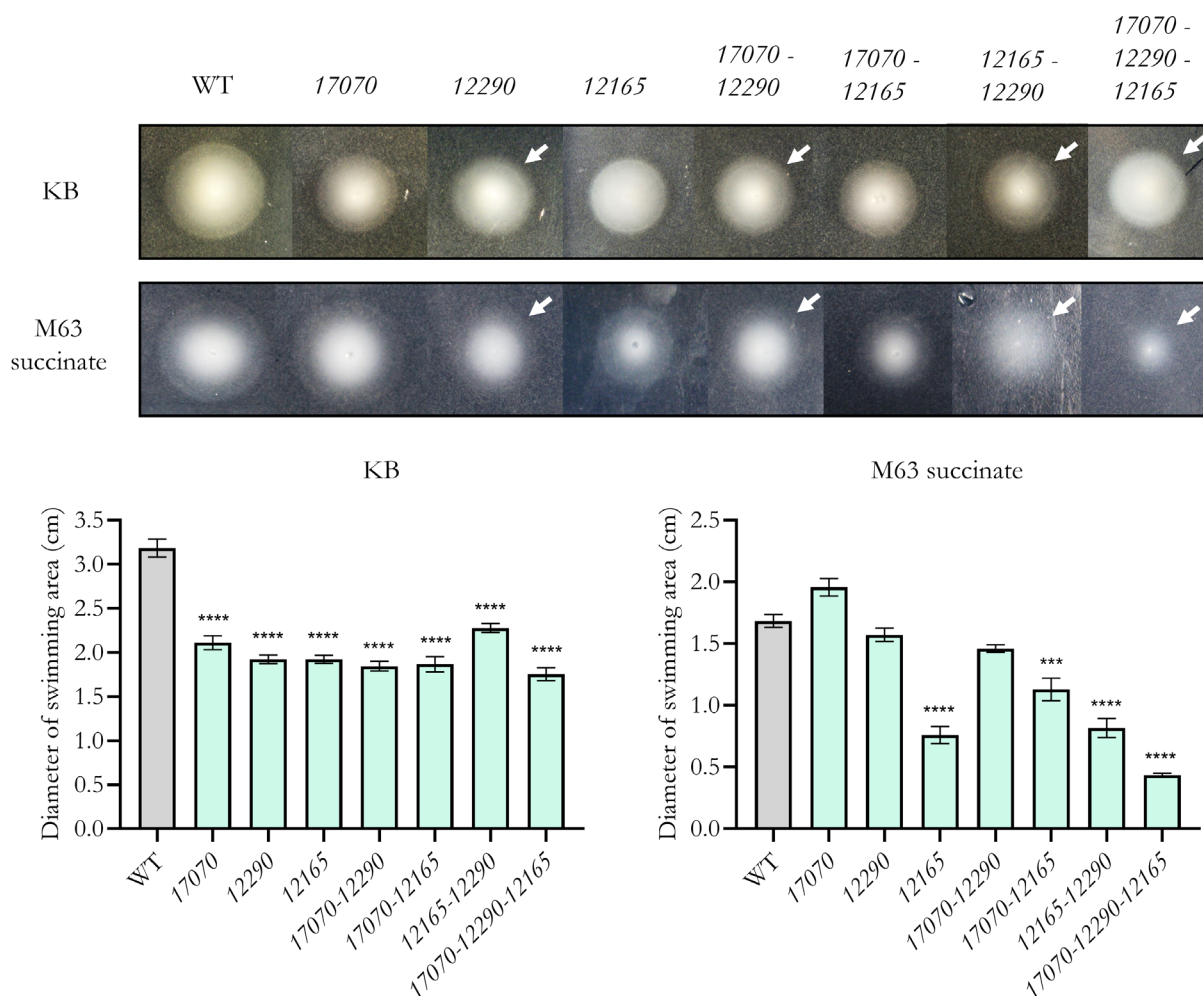


**Figure 36. Swimming motility of *Aer*-like mutant strains in KB and M63 succinate media.** Shown are means and standard errors four independent experiments with four technical replicates each. Values that are significantly different are indicated by asterisks (\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

### 3.2.3. *Aer*-like chemoreceptors mediate taxis to atmospheric air

We performed air-trap assays to assess the capacity of mutant strains to perform taxis towards atmospheric air (Figure 37). This assay consists in creating an air-trap inside a Pasteur pipette, where oxygen concentration is higher. This way, bacteria are forced to actively move towards the trap to achieve optimal concentrations of oxygen. The 17070-12290 double mutant was the only strain that showed significantly less accumulation near the air.

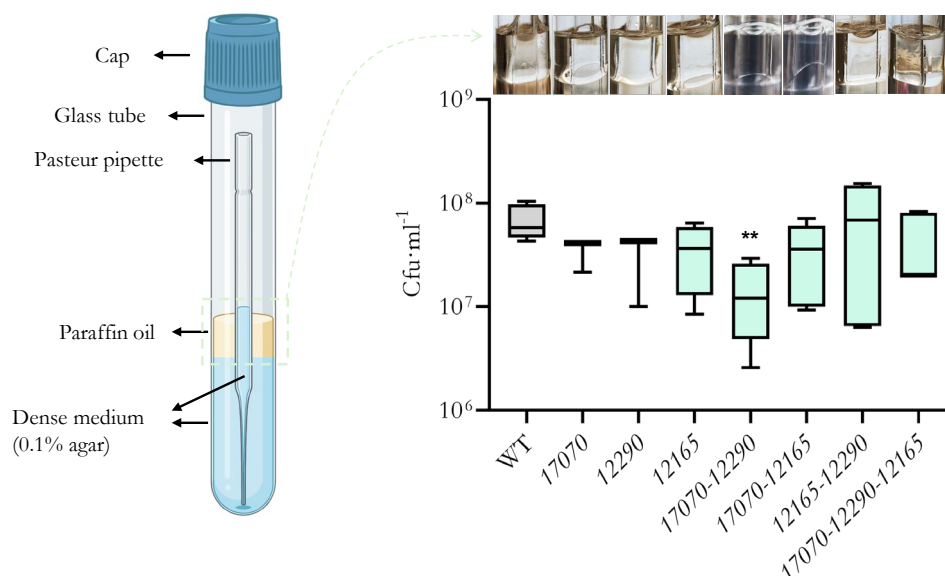


**Figure 37. Air trap assays of *Aer*-like mutant strains.** A schematic representation of the experimental setup is shown on the left side of the figure. Bacterial counts expressed in  $\text{cfu}\cdot\text{ml}^{-1}$  are shown on the right side of the figure. Shown are means from three independent experiments with three technical replicates each. Values that are significantly different are indicated by asterisks (\*\* $p < 0.01$ ).

### 3.2.4. *Aer*-like chemoreceptors are involved in biofilm formation

We carried out attachment assays to determine the role of energy taxis transducers in regulating biofilm lifestyle. We used the crystal violet method with the SOBG medium, used previously in Dd3937 (Manjurul Haque et al., 2012) (Figure 38).

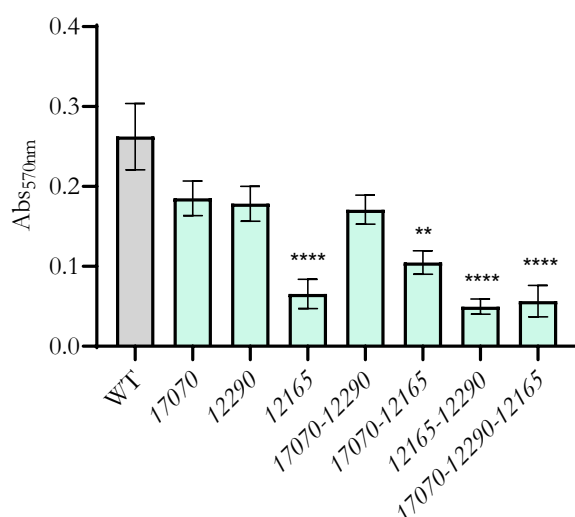


**Figure 38. Crystal violet attachment assays in Dd3937 strains.** Shown are means and standard errors from three independent biological replicates with eleven technical replicates each. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\*\*\* $p < 0.0001$ ).

We observed how all strains carrying a mutation in the DDA\_RS12165 chemoreceptor displayed significantly less attachment in comparison to the WT. Regarding the remaining strains tested,

mutations in the DDA\_RS17070 and DDA\_RS12290 chemoreceptors also seem to slightly reduce attachment capacity, but differences were not statistically significant.

### 3.2.5. Aer-like chemoreceptors from Dd3937 are involved in infection progression of potato plants

To analyze the importance of energy taxis in the context of plant pathogenesis, we decided to assess the *Aer*-like mutant strains in their capacity to both enter and colonize leaves of potato plants (Figure 39).

On one hand, mutant strains *12165* and *17070-12290-12165* showed significantly reduced capacity to enter potato leaves at 24 h post drop-inoculation. On the other hand, all strains carrying a DDA\_RS12165 mutation displayed a significant reduced capacity to colonize the apoplast of potato leaves. Although statistically significant, these differences were very subtle and should be taken with care.

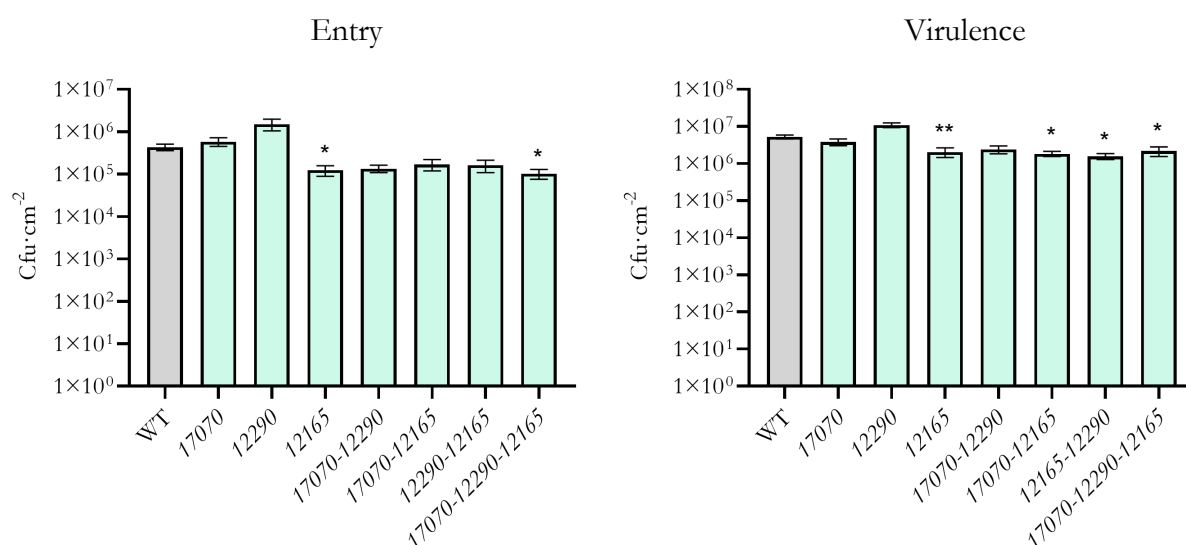


**Figure 39. Entry and virulence assays in potato plants.** (A) Bacterial counts in entry assays expressed in cfu·cm<sup>-2</sup>. Bacterial entry was determined based on bacterial population sizes at 24 h post drop-inoculation ( $5 \times 10^7$  cfu·ml<sup>-1</sup>). (B) Bacterial counts in virulence assays expressed in cfu·cm<sup>-2</sup>. Virulence was assessed based on bacterial population sizes in potato leaves at 24 h after syringe-infiltration ( $5 \times 10^7$  cfu·ml<sup>-1</sup>). Shown are means and standard errors from three independent biological replicates. In each experiment, three plants were analyzed by sampling five 1-cm-diameter leaf discs of each strain per plant. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ ).

Therefore, we decided to assess the overall virulence capacity by analyzing the macerated area potato leaves at 24 h post drop-inoculation (Figure 40AB). Moreover, we also determined the percentage of successful infection initiation (Figure 40C).

Potato leaves infected with all mutants displayed significantly lower macerated area, except for the triple *17070-12290-12165* mutant. Similar results were obtained when we looked at the capacity of each strain to initiate infection. All strains but the triple *17070-12290-12165* mutant decreased their capacity to initiate infection, although statistically significant differences were only obtained for the simple *17070* and *12290* mutants, and the double *17070-12165* and *12165-12290* mutants.

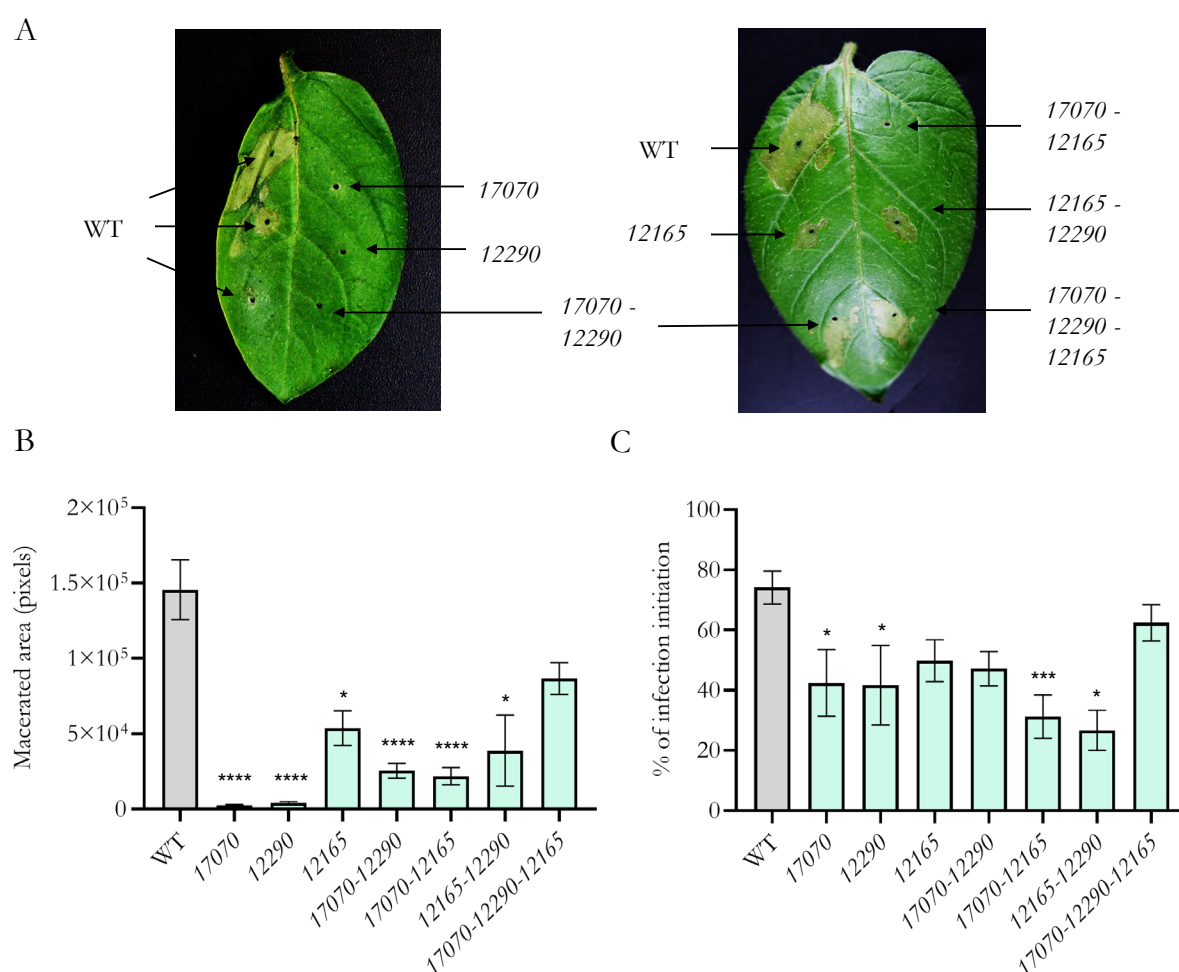


**Figure 40. Symptom development in potato plants.** (A) Symptom development in potato leaves. (B) Measurement of macerated area. (C) Evaluation of the percentage of initial successful infections. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

### 3.3. Discussion

Energy taxis is a key strategy for survival of phytopathogenic bacteria, enabling them to locate preferred niches for energy generation (Schweinitzer & Josenhans, 2010; Xie et al., 2010). Moreover, sensing the internal energy status is a more versatile sensory response than directly perceiving oxygen (Taylor et al., 2001). It is known that chemotaxis plays a crucial role in the pathogenicity of Dd3937 (Antúñez-Lamas et al., 2009; Gálvez-Roldán et al., 2023; Río-Alvarez et al., 2014). However, energy taxis and its role in virulence has not yet been studied in this bacterium. Thus, with this work, we aimed to characterize three chemoreceptors putatively involved in energy taxis, DDA3937\_RS17070, DDA3937\_RS12290, and DDA\_RS12165, and their role in plant pathogenesis.

First of all, it is important to point out that only four out of the 47 chemoreceptors of Dd3937 contain PAS domains and, therefore, are putatively involved in energy taxis. Notably, in other bacteria, this number is also quite reduced. Moreover, studies have experimentally proven how aerotaxis receptors from other bacteria support energy taxis in *E. coli*, which manifests their function homology in different bacteria (Jian & Caitlyn, 2007).

We started the Dd3937 energy taxis characterization observing the growth capacity of the *Aer*-like mutant strains in rich and minimal media with succinate as a sole carbon source. From the obtained results, we observed that DDA\_RS17070 and DDA\_RS12290 do not seem to have a role in supporting optimal growth in any of the media tested. However, all strains tested displayed a smaller swimming diameter in rich media. In *A. brasilense*, the efficiency of a chemical as a growth substrate correlates directly with its efficiency as chemoeffector, as well as with the increase in the intracellular energy level upon substrate addition (Alexandre et al., 2000). Since the simple 17070, 12290, and the double 17070-12290 strains did not show a growth defect in rich media, DDA\_RS17070 and DDA\_RS12290 might be regulating swimming motility uncoupled from growth in these conditions. In the case of the 12165 and the 17070-12290-12165 mutants, we cannot rule out that the defect observed in swimming is aggravated because of their slower growth rate both in rich media and minimal media supplemented with succinate. These two phenotypes could also explain the reduced capacity exhibited by these two strains to enter potato leaves, where the nutritional conditions are also minimal.

Succinate is a central metabolite of the TCA cycle and its conversion to fumarate reduces two FAD molecules. As mentioned in the introduction of this chapter, FAD perception in its different forms by *Aer*-like chemoreceptors regulates flagellar rotation. Therefore, one hypothesis could be that a mutation in DDA\_RS12165 impedes the strains to optimally perceive the different oxidative states of FAD, causing the swimming phenotype of strains carrying a DDA\_RS12165 mutation when growth relies on succinate alone. In fact, we tested the chemoattraction of Dd3937 towards succinate in capillary assays, in which chemoattraction is uncoupled from growth because the incubation time is limited to 30 minutes (Figure S10). Here, we observed how Dd3937 displayed a typical

chemoattraction pattern. This suggests the existence of a chemoreceptor with a periplasmic LBD that responds to the presence of succinate in the environment. It would be interesting to test the *Aer*-like mutants in capillary assays against succinate. If no difference was observed between the WT and the mutant strains in this assay, we could prove that the swimming defect is exclusively due to a change in energy perception.

One of the main roles of energy taxis is to enable bacteria to navigate in a spatial oxygen gradient in search of optimum concentration for energy generation. This specific form of energy taxis has been termed aerotaxis (Arrebola & Cazorla, 2020). Although Dd3937 is a facultative anaerobe, aerobic respiration is preferred, and aerotaxis-driven motility could direct bacteria towards the optimal oxygen concentration for energy generation. We could assess the aerotactic behavior of our mutant strains in both swimming motility assays and air trap experiments. In both media tested, strains carrying a *DDA\_RS12290* mutation lost the ability to accumulate in oxygen rich locations. This phenotypic trait was not observed in any other strain, which clearly indicates that chemoreceptor DDA\_RS12290 has a specific role either in oxygen perception or in supporting aerobic metabolism. This is supported by the phenotype observed for the *17070-12290* strain in air trap assays. However, no phenotype was observed for the remaining strains, even if they carried a *12290* mutation. The difference observed between experiments could be because oxygen diffusion might be lower in soft agar, emphasizing the role of aerotaxis in moving towards the oxygen gradient. Therefore, Dd3937 could be facing a more challenging environment in this assay, revealing a defect in motility towards oxygen in the single *12290* mutant.

As previously mentioned across this manuscript, biofilm and motility are frequently regulated inversely in bacteria. Following this rule, we hypothesized that our mutant strains would have increased biofilm formation capacity. However, this hypothesis is not supported by the decrease in attachment of all strains carrying a *DDA\_RS12165* mutation. Interestingly, biofilm formation has been observed to be regulated by aerotaxis receptors in other bacteria, although with some controversy. In some bacteria, aerotaxis receptors act as positive regulators of biofilm formation (Arrebola & Cazorla, 2020; Hölscher et al., 2015; Tumewu et al., 2022), while in other bacteria act as negative regulators (Jian & Caitilyn, 2007; Jiang et al., 2016). Our work suggests that DDA\_RS12165 could act as a positive regulator, as any strain containing this mutation decreases its capacity to attach to surfaces. As discussed in chapter I, high attachment and biofilm formation correlates with high levels of c-di-GMP. In Dd3937, the second messenger c-di-GMP has been described to play a central role in virulence regulation, downregulating expression of genes encoding CWDEs (Yuan et al., 2020). Since Dd3937 experiments low oxygen availability in plant tissue (Hugouvieux-Cotte-Pattat et al., 1992; Reverchon et al., 2016), and the expression of important virulence factors such as pectate lyase is induced under anaerobic conditions (Hugouvieux-Cotte-Pattat, 2016), we hypothesized that energy taxis might be essential in the regulation of the plant infection process. Assays in *Solanum tuberosum* show how all

mutant strains but the triple *17070-12290-12165* mutant strain are unable to macerate the tissue compared to the WT strain. This reduced maceration capacity could be due to a reduced ability in sensing the micro aerobiosis conditions in plant tissues which are responsible of the induction of CWDEs activity during infection (Yuan et al., 2020). One hypothesis to explain the high macerating phenotype of the triple *17070-12290-12165* mutant is that the expression of CWDEs could be constitutively active because the lack of Aer-like chemoreceptors is preventing the strain from perceiving the environment in terms of oxygen levels.

In addition, it has been recently shown how the levels of tricarboxylic acid cycle intermediates are used as a signal to coordinate gene expression via c-di-GMP (Yuan et al., 2020). It would not be surprising to find that c-di-GMP plays a major role in aerotaxis regulation in Dd3937. In fact, the link between this second messenger and aerotaxis has been reported in *A. brasilense*, proving that c-di-GMP binds to the CR Tlp1 modulating its signaling function during aerotaxis (O’Neal et al., 2017; Russell et al., 2013).

All these results strongly point to a link between the perception of the inner metabolic state and gene expression, especially virulence related genes. Therefore, experiments to look at gene expression are crucial to understand these phenotypes and how energy taxis regulates virulence related traits.

## **General discussion**



Chemoperception is crucial for bacteria to interact with their surrounding environment. Throughout this manuscript, we have discussed bacterial chemoperception from many perspectives. We have covered external perception linked to chemotaxis, processing of the signal through multiple chemosensory systems, and internal perception linked to energy taxis. There are numerous examples of how both these processes are crucial for a successful infection in plant pathogenic bacteria (Ant  nez-Lamas et al., 2009; Cerna-Vargas et al., 2019; Hawes & Smith, 1989; Hida et al., 2015; Jian & Caitilyn, 2006, 2007; Kumar Verma et al., 2018; Malamud et al., 2011; Matas et al., 2012; Santamar  a-Hernando et al., 2020; Tumewu et al., 2020). However, many research questions remain still open on each one of these steps. Knowledge on the specific roles of the different systems in bacteria with multiple chemosensory systems is still scarce. Moreover, information regarding specific plant signals and their effect on chemotaxis and virulence is also limited. Finally, the field of energy taxis and its role in plant pathogenesis is largely unexplored.

In this thesis, we tackle these issues in two model plant pathogens with very different lifestyles. First, we have performed a deep functional characterization of three of the four chemosensory systems of *Pseudomonas syringae* pv. tomato DC3000 (PsPto). Second, we characterized one chemoreceptor from PsPto involved in GABA and L-Pro perception, which are two molecules linked to plant immunity. And third, we have analyzed three chemoreceptors from *Dickeya dadantii* 3937 (Dd3937) putatively involved in energy taxis and its role in plant pathogenesis.

## 1. Chemosensory systems of PsPto and Dd3937

Dd3937 presents 47 chemoreceptors, and even though they are of different heptad classes, they all feed into a single chemosensory system categorized as F7. In contrast, PsPto presents 49 chemoreceptors, feeding into four different chemosensory systems categorized as F6, F8, ACF, and Tfp. We have been able to visualize the chemoreceptor arrays derived from the F6 and F8 systems of PsPto. We have not conducted these experiments with Dd3937 since it was out of the scope of this investigation. However, F7 chemoreceptor arrays have been successfully imaged through cryo-electron tomography in other bacterial species like *Escherichia coli* (Briegel et al., 2009, 2012; Liu et al., 2012; Zhang et al., 2007), *Salmonella enterica* (Briegel et al., 2012), *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Shewanella oneidensis*, or *Methylomicrobium alcaliphilum* (Ortega et al., 2020). Among these species there are those that contain a sole F7 chemosensory system (*E. coli* and *S. enterica*), like Dd3937; and those that contain an F7 chemosensory system in addition to other systems (*P. aeruginosa*, *V. cholerae*, *S. oneidensis*, and *M. alcaliphilum*). Notably, not all F7 systems looked similar in all these species. In species with a single F7 system, the arrays looked similar to those from the F6 system, which are linked to the membrane and have a height of around 25 nm approximately.

Moreover, they exhibited a layer of periplasmic domains just outside the inner membrane. However, F7 arrays present in bacteria with multiple chemosensory system were taller (40 nm approximately) and lacked discernable periplasmic densities (Ortega et al., 2020). This depends entirely on the chemoreceptors comprising the array. All chemoreceptors from *E. coli*, *S. enterica* or *D. dadantii* will associate with their F7 system, while only one or a few chemoreceptors have been determined to comprise the F7 array in bacteria with multiple chemosensory systems. Previous studies have determined that different evolutionary stages (1 to 5) occurred in the transition from F6 to F7 systems (Ortega et al., 2020). Thereby, the F7 systems from *P. aeruginosa* or *S. oneidensis* have been categorized as stage 1 and stage 2, which represent the most ancient ones, in which flagellar functions still belong to F6 systems, which they also present. However, bacteria evolved to eliminate F6 systems and transferred flagellar functions to F7 systems. This is believed to have happened in stages 3 and 4, where some features from the F6 systems still remain, but they are not entirely functional. Finally, bacteria containing stage 5 F7 systems completely eliminate the presence of F6 proteins, which is the case of *E. coli*, *S. enterica* and *D. dadantii*. Here, flagellar functions are completely transferred and, in the case of Dd3937, this has been experimentally proven by Antúñez-Lamas et al. (2009), where mutations in F7 key proteins resulted in impaired motility.

In contrast to Dd3937, PsPto contains four different chemosensory systems (F6, F8, ACF, and Tfp). We have been able to visualize the F6 and F8 systems, and we could identify them based on height prediction of chemoreceptors they could associate with. Following this rationale, we determined how PSPTO\_0912, the only chemoreceptor predicted to feed into this system, forms the F8 array, with a height of 20 nm. Notably, genomic organization of the F8 system is highly similar to that of the stage 1 and stage 2 F7 systems. For instance, the only difference between the F7 system from *P. aeruginosa* and the F8 system from PsPto is the presence of a STAS-domain in between CheY and CheA in PsPto, and the change of location of CheW, which moves one position to locate downstream of CheA in PsPto. Moreover, the presence of two chemoreceptors (usually named McpA and McpB) is conserved between these two systems, and the presence of a C-terminal pentapeptide is also conserved in the McpB, which corresponds to PSPTO\_0912. However, LBD topology and overall structure of the MCPs differ between systems. While the McpB from *P. aeruginosa* is cytoplasmic and contains a PAS domain, the one from PsPto (PSPTO\_0912) is transmembrane and contains a 4HB LBD. This suggests a close evolutionary relationship between F8 systems with stage 1 and 2 F7 systems. Interestingly, these kind of F7 systems were only visible through cryo-ET when bacteria were exposed to stress conditions (Ortega et al., 2020), which suggests their role is restricted to very specific conditions. This is not the case for the F8 system from PsPto, which we were able to observe frequently in PsPto when cultivated in rich medium, which could indicate a strong constitutive role of the F8 system in PsPto. Here, we have determined the role of the histidin kinase (HK) and response regulator (RR) from this system in pathogenicity related traits like motility, biofilm formation, and

virulence. We showed how HK and RR do not seem to work together in regulating swimming motility, twitching, and biofilm, as mutants had opposite phenotypes in several experiments. Since PSPTO\_0912 is the only chemoreceptor predicted to compose the F8 array, we decided to assess the swimming motility of a strain lacking this chemoreceptor ( $\Delta 0912$ ) in rich media, where F8 arrays are known to be present (Figure S11). This strain presented a WT-like swimming motility, supporting the idea that this chemoreceptor and this array have no role in directly regulating swimming motility. In order to confirm that F8 array is solely formed by PSPTO\_0912 proteins, we should carry out cryo-electron microscopy experiments with the  $\Delta 0912$  strain. Our BACTH assays revealed that PSPTO\_0912 could interact with kinases from the F6 and ACF systems as well. Identifying potential ligands from PSPTO\_0912 would be crucial for unveiling the role that PSPTO\_0912 might have in PsPto.

This work provided a broad characterization of the F and ACF chemosensory systems of PsPto, proving that kinases and response regulator do not always work together, and that crosstalk exists between the systems. We unveiled this crosstalk at the level of CheA1 and CheA2 kinase interaction with the response regulator of the ACF system, WspR. A similar situation occurs in *Comamonas testosteroni*, where the chemotaxis CheA kinase also interacts with the response regulator from a biofilm controlling chemosensory system (Huang et al., 2019). The nature of each cluster is different from those of PsPto, but crosstalk still occurs. Even though chemoreceptor and cluster separation in different arrays has been seen as a potential form to prevent crosstalk (Wadhams & Armitage, 2004), it seems it occur regardless *in vivo*.

PsPto and Dd3937 differ greatly in the number of chemoreceptors containing a C-terminal pentapeptide. Ortega & Krell (2020) found that bacteria that maintained host interactions had more abundance of chemoreceptors with pentapeptides than others. For instance, the highest abundance in chemoreceptors with C-terminal pentapeptide are those that inhabit the human gut. Only the PSPTO\_0912 chemoreceptor contains such feature in PsPto, while 19 chemoreceptors from Dd3937 contain a C-terminal pentapeptide. This peptide interacts with specific CheR proteins, tethering the CheR closer to the methylation sites, fine tuning adaptation of the chemotaxis system (Le Moual et al., 1997; Li & Hazelbauer, 2005). Since not all chemoreceptors present this feature, one hypothesis could be that the recruitment of CheR can be mediated only by one or a few chemoreceptors with C-terminal pentapeptides, allowing adaptation of all chemoreceptors from the same array. This could be the case of Dd3937, in which all chemoreceptors must form one single array. However, PsPto supposedly forms an F8 array solely on PSPTO\_0912 chemoreceptors, which would physically make it impossible for mediating adaptation of all chemoreceptors present in the F6 array. Following this hypothesis, one could think that PSPTO\_0912 also takes part in the F6 array, which would be supported by the interaction observed between PSPTO\_0912 and CheA2 in BACTH assays. Nevertheless, as mentioned

above, no swimming defect was observed in strains lacking this chemoreceptor. If this chemoreceptor was in charge of recruiting CheR for the rest of CRs, a failure or at least a defect in the swimming capacity of  $\Delta 0912$  would be expected.

## 2. Chemotaxis and metabolism

In 1969, Julius Adler provided for the first time biochemical and genetic evidence that chemotaxis to a certain stimulus is independent from uptake or metabolism of such stimulus in *E. coli*. For example, attraction to galactose was normal in mutants deficient in galactose transport and metabolism. In contrast, some chemotactic responses require metabolism of such attractant, what we call energy taxis. Some examples in *E. coli* are chemotaxis to proline (Clancy et al., 1981), glycerol (Zhulin et al., 1997), and succinate (Bibikov et al., 1997). In the case of chemotaxis to glycerol, mutants defective on its metabolism lost their attraction to this carbon source (Zhulin et al., 1997). In this work we have characterized PsPto-PscC as a GABA and L-Pro chemoreceptor. It is important to remind that GABA is the most abundant amino acid in the tomato apoplast (Rico & Preston, 2008). Even though we demonstrated that PsPto-PscC binds GABA and L-Pro directly through its LBD, we cannot rule out that GABA and L-Pro chemoperception elicit an energy taxis response.

Proline chemotaxis was found to be linked to its metabolism by Clancy et al. (1981) in *E. coli*. Chemotaxis to proline was only present when bacteria had been exposed to proline as a growth substrate. Moreover, chemotaxis to proline was abolished in the presence of succinate as an additional metabolizable substrate. Authors claim that proline chemotaxis results from a direct impact of proline oxidation on the metabolism of the bacterial cell. Similarly, chemotaxis to proline was also suggested to be dependent on its metabolism in *Sinorhizobium meliloti*, a nitrogen-fixing symbiont of leguminous plants (Gotz et al., 1982). This was observed because chemotactic responses of *S. meliloti* were stronger when cells had been starved before being chemotactically stimulated.

PsPto contains a large number of genes for the conversion of GABA to succinic semialdehyde (*gabT1*, *gabT2*, and *gabT3*), and the subsequent conversion of succinic semialdehyde to succinate (*gabD1*, *gabD2*, and *gabD3*) (McCraw et al., 2016). Park et al. (2010) noted that this high amount of GABA metabolism genes in comparison to other pseudomonads might imply “an exceptional ability to convert GABA to succinate”. In this work, we linked GABA chemoperception through the PsPto-PscC chemoreceptor to changes in gene expression of a subset of the above-mentioned GABA metabolism genes. Briefly, we found how *gabT1*, *gabT2* and *gabD2* were downregulated in a PsPto-*pscC* mutant in the presence of GABA. Chemoperception and subsequent catabolism of GABA could be generating succinate, therefore stimulating the TCA cycle. This would generate FAD, which could in turn stimulate the PAS domain-containing chemoreceptors from PsPto. To determine if GABA

chemoperception is dependent on metabolism, it would be interesting to assess the ability to perceive GABA in mutants defective in the *gadT* and *gadD* genes.

High concentrations of GABA repress expression of T3SS genes in PsPto. It has been hypothesized that the repressive effect of GABA on virulence, together with the high abundance of GABA metabolism genes, could mean that PsPto needs to degrade GABA rapidly to lessen the repressive effect (Park et al., 2010; Yu et al., 2013). The repression of *hrpL* was observed to be greater in PsPto strains defective on GABA metabolism, which indicates that this regulation is not consequential of GABA catabolism, but of the presence of GABA itself. We could hypothesize that PsPto metabolizes GABA quickly to avoid its repressive effect on T3SS expression, but also to stimulate energy taxis and improve its capacity to colonize the plant tissue.

This is not the first time that a chemoreceptor in principle not involved in energy taxis influences metabolism of its attractant. The *Dd15070* chemoreceptor from Dd3937 binds nitrite and nitrate directly (Gálvez-Roldán et al., 2023) and has been shown to be involved in virulence in potato plants. In this case, a mutation in this chemoreceptor caused an upregulation of an assimilatory nitrate reductase. Authors claim that chemoreceptor Dd15070 plays a role in regulating nitrate assimilation. This is just another example of how a chemoreceptor that had no putative role in energy taxis has an influence in bacterial metabolism, suggesting that chemotaxis and energy taxis are interlaced processes.

It would be very interesting to couple research on chemoreceptors and chemotaxis of specific metabolizable compounds with studies on metabolism of these specific effectors, as it would provide a global overview of chemotaxis and energy taxis. Some work has been done in this direction, mainly in the chemotaxis model bacteria *E. coli* and *Bacillus subtilis*. Yang et al. (2015) investigated the relation between amino acid consumption and its chemotaxis. Interestingly, the results were very different between the two bacteria. In *E. coli*, the vast majority of amino acids that were attractants were also consumed during growth. Moreover, those that elicited a stronger chemotactic response were the first to be consumed. In contrast, *B. subtilis* was attracted to a broader spectrum of amino acids regardless of its consumption as a nutrient. The authors hypothesize that this difference must be linked to their habitat and how these bacteria use chemotaxis for different purposes (Yang et al., 2015b).

It is incredibly interesting how bacteria are so different in their number of chemoreceptors, as well as in their number of chemosensory systems. We have shed some light on how these systems interplay in *Pseudomonas syringae* pv. tomato DC3000, as well as characterizing one chemoreceptor involved in the interaction of this bacterium with tomato plants. We also explored the role of energy taxis in *Dickeya dadantii* 3937, proving its importance in virulence. Hopefully, this thesis will inspire other scientists to follow up on these intriguing topics.



## Conclusions



1. We assigned 48 out of 49 chemoreceptors of *Pseudomonas syringae* pv. tomato DC3000 (PsPto) to chemosensory systems. Our experimental data suggests that chemoreceptors can associate with more than one kinase. Therefore, more experimental work is needed to corroborate this assignment.
2. We successfully visualized the F6 and F8 chemosensory arrays in PsPto through cryo-electron microscopy.
3. F6 governs chemotaxis and swimming motility in PsPto, while the F8 and ACF systems have a role in modulating swimming motility. Kinase and response regulator from the F6 system work together to regulate chemotaxis and swimming motility. In contrast, kinase and response regulator from the F8 system do not work together to regulate swimming motility. In fact, CheY1 has no role in modulating flagellar motility.
4. The F and ACF systems are also involved in the regulation of biofilm formation and twitching motility. Kinase and response regulators from the F systems do not work together in regulating these traits.
5. CheA1, CheA2, and CheA3 interact with WspR, proving there is crosstalk among chemosensory systems at the level of interaction with response regulators.
6. F6, F8, and ACF chemosensory systems are important for plant pathogenesis. More specifically, the ACF system seems to play a role in symptom development.
7. PsPto-PscC chemoreceptor binds GABA and L-Pro and mediates chemotaxis towards these two compounds. Its chemoperception regulates the expression of the GABA catabolism genes *gabT1*, *gabT2* and *gabD2*. However, it does not control the expression of T3SS related genes.
8. PsPto-PscC is required for full virulence in tomato plants.
9. DDA\_RS12165 is necessary to support optimal growth in *Dickeya dadantii* 3937 (Dd3937)
10. DDA\_RS12290 is presumably involved in the perception of O<sub>2</sub> in Dd3937.
11. The three Aer-like chemoreceptors of Dd3937 are involved in symptom development during the potato infection process.



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## **Annexes I**



## Supplementary Tables

**Table S1. Chemoreceptors of *Pseudomonas syringae* pv. tomato DC3000.**

| Chemosensory system | MCP        | LBD-domain  | Class (Heptads) | C-terminal pentapeptide | Transmembrane domain |
|---------------------|------------|-------------|-----------------|-------------------------|----------------------|
| F6                  | PSPTO_0466 | 4HB_MCP_1   | 40H             | -                       | Yes                  |
|                     | PSPTO_0995 |             |                 |                         |                      |
|                     | PSPTO_1334 |             |                 |                         |                      |
|                     | PSPTO_2997 |             |                 |                         |                      |
|                     | PSPTO_3279 |             |                 |                         |                      |
|                     | PSPTO_3291 |             |                 |                         |                      |
|                     | PSPTO_3577 |             |                 |                         |                      |
|                     | PSPTO_3580 |             |                 |                         |                      |
|                     | PSPTO_3685 |             |                 |                         |                      |
|                     | PSPTO_4541 |             |                 |                         |                      |
|                     | PSPTO_4624 |             |                 |                         |                      |
|                     | PSPTO_5569 |             |                 |                         |                      |
|                     | PSPTO_1061 |             |                 |                         |                      |
|                     | PSPTO_2448 |             |                 |                         |                      |
|                     | PSPTO_2480 |             |                 |                         |                      |
|                     | PSPTO_3237 |             |                 |                         |                      |
|                     | PSPTO_1059 |             |                 |                         |                      |
|                     | PSPTO_2616 |             |                 |                         |                      |
|                     | PSPTO_3098 |             |                 |                         |                      |
|                     | PSPTO_3680 |             |                 |                         |                      |
|                     | PSPTO_5159 |             |                 |                         |                      |
|                     | PSPTO_5160 |             |                 |                         |                      |
|                     | PSPTO_5553 |             |                 |                         |                      |
|                     | PSPTO_0263 |             |                 |                         |                      |
|                     | PSPTO_0916 |             |                 |                         |                      |
|                     | PSPTO_2475 |             |                 |                         |                      |
|                     | PSPTO_3480 |             |                 |                         |                      |
| PSPTO_3379          |            |             |                 |                         |                      |
| PSPTO_3699          |            |             |                 |                         |                      |
| PSPTO_1066          |            |             |                 |                         |                      |
| PSPTO_2254          |            |             |                 |                         |                      |
| PSPTO_2472          |            |             |                 |                         |                      |
| PSPTO_2526          |            |             |                 |                         |                      |
| PSPTO_4936          |            |             |                 |                         |                      |
| PSPTO_5554          |            |             |                 |                         |                      |
| PSPTO_1648          |            |             |                 |                         |                      |
| PSPTO_2014          |            |             |                 |                         |                      |
| PSPTO_4531          |            |             |                 |                         |                      |
| PSPTO_2511          |            |             |                 |                         |                      |
| PSPTO_0117          |            |             |                 |                         |                      |
| PSPTO_1008          |            |             |                 |                         |                      |
| PSPTO_5352          |            |             |                 |                         |                      |
| PSPTO_3753          |            |             |                 |                         |                      |
| PSPTO_4786          |            |             |                 |                         |                      |
| PSPTO_2883          |            |             |                 |                         |                      |
| F8                  | PSPTO_0912 | 4HB_MCP_1   | 34H             | EFTRF                   | Yes                  |
| ACF                 | PSPTO_1493 | 4HB_MCP_1   | 40H             | -                       | Yes                  |
| TFP                 | PSPTO_5031 | PilJ        | 40H             | -                       | Yes                  |
| Unassigned          | PSPTO_2441 | (PAS_3) x 4 | 36H             | -                       | No                   |

Table S2. Number of *Pseudomonas syringae* pv. tomato cells with chemotaxis arrays.

|                | F6 arrays | F8 arrays | F6 & F8 arrays |
|----------------|-----------|-----------|----------------|
| WT             | 15        | 6         | 1              |
| $\Delta cheA1$ | 15        | 0         | 0              |
| $\Delta cheA2$ | 13        | 8         | 4              |

## Supplementary Figures

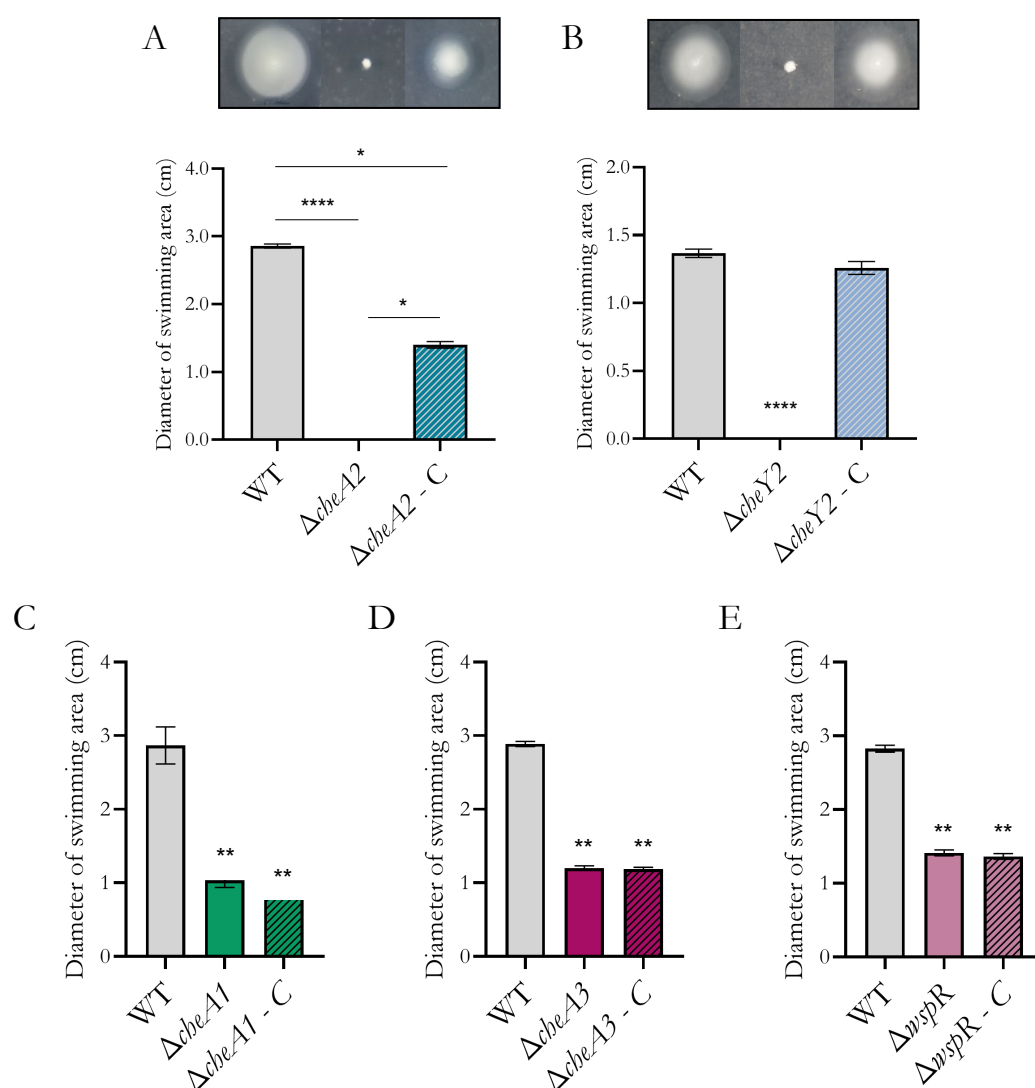


**Figure S1. Swimming motility assays of WT, chemosensory systems mutants and complemented strains.** Assays were performed in M9 minimal media supplemented with 10 mM citrate and incubated for 48 (B) or 72 hours (A, C, D, E). Shown are means and standard errors of three independent experiments with four technical replicates each. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ ).



**Figure S2.** Representative image of the inner morphology displayed by colonies grown on KB supplemented with Coomassie and Congo Red. Experiments were conducted in triplicate with six technical replicates each.

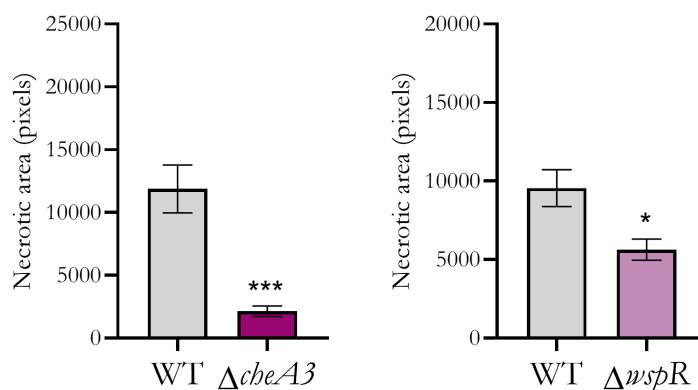


**Figure S3.** Symptom development in tomato leaves by the WT,  $\Delta cheA3$ , and  $\Delta wspR$  strains. Shown are means and standard errors of the necrotic areas measured in tomato leaves. Three independent experiments were conducted with three technical replicates each. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\*\* $p < 0.005$ ).

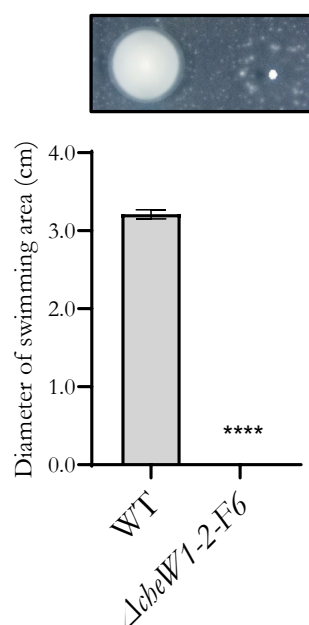


**Figure S4.** Swimming motility assay of  $\Delta cheAW-F6$  mutant strain in M9 10 mM citrate. Shown are means and standard errors of three independent experiments with four technical replicates each. Values that are significantly different are indicated by asterisks (\*\*\*\* $p < 0.0001$ ).

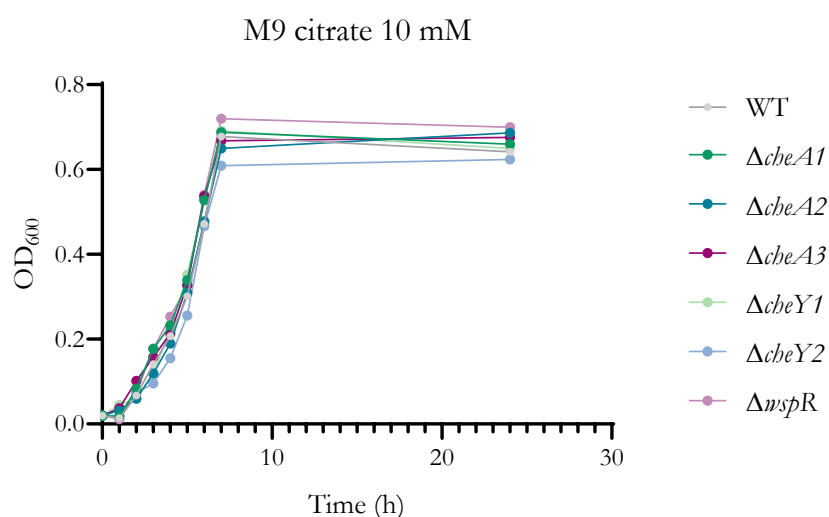


**Figure S5. Growth curve of WT and kinase and response regulator mutant strains in M9 minimal medium supplemented with 10 mM citrate.** Measurements were performed each hour for 7 h and a final point at 24 h.

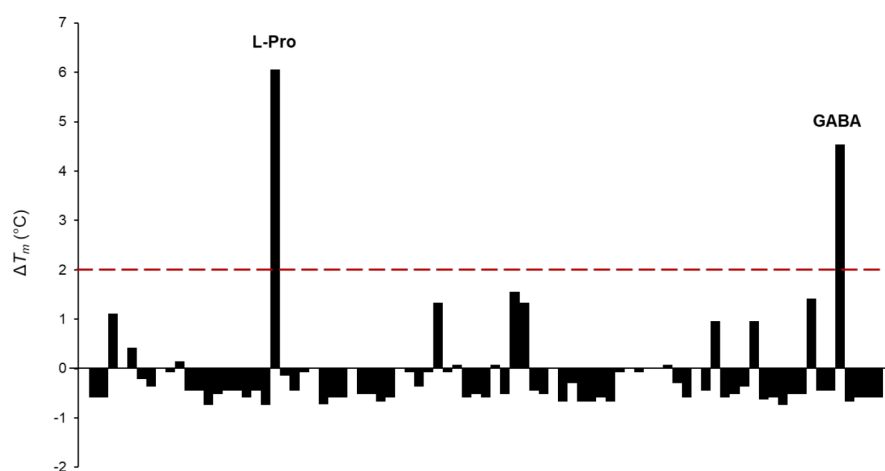


**Figure S6. Differential scanning fluorimetry-based ligand screening of PsPto-PscC-LBD.** Shown are the changes in the midpoint of the protein unfolding transition ( $T_m$ ) for the compounds present in the Biolog PM3B phenotype microarray of nitrogen sources with respect to the  $T_m$  of the ligand-free protein. The dashed line indicates the threshold of 2°C for significant hits.

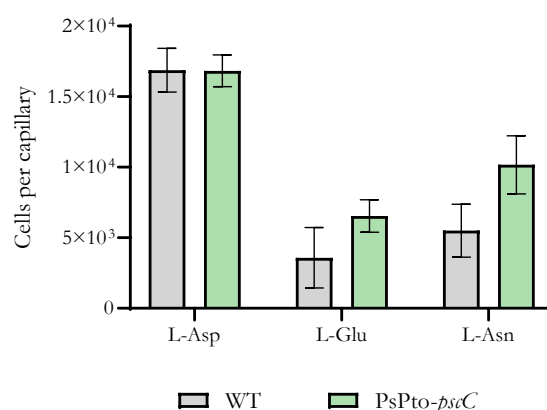


**Figure S7. Chemotaxis capillary assays of *Pseudomonas syringae* pv. tomato (WT) and the PsPto-*pscC* mutant towards L-Asp, L-Glu, and L-Asn at 1 mM.** The data have been corrected with the number of cells that swam into buffer-containing capillaries. Shown are means and standard errors from three independent experiments.

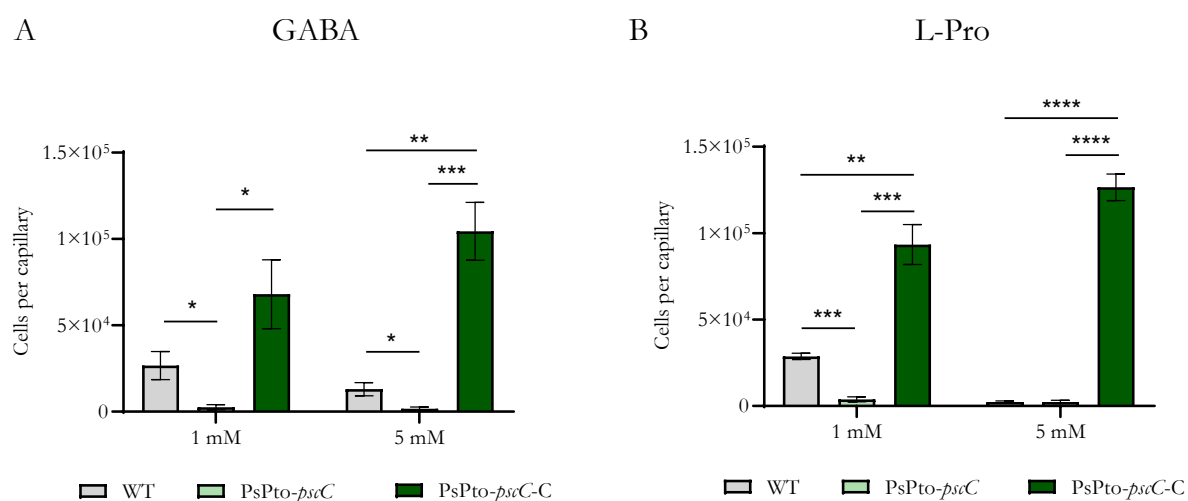


**Figure S8. Quantitative capillary chemotaxis assays of *Pseudomonas syringae* pv. tomato (WT), the PsPto-*pscC* mutant, and the complemented mutant (PsPto-*pscC*-C) towards GABA and L-Pro at 1 and 5 mM.** The data have been corrected with the number of cells that swam into buffer-containing capillaries. Shown are means and standard errors from three independent experiments conducted in triplicate. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.005$ ; \*\*\*\* $p < 0.001$ ).

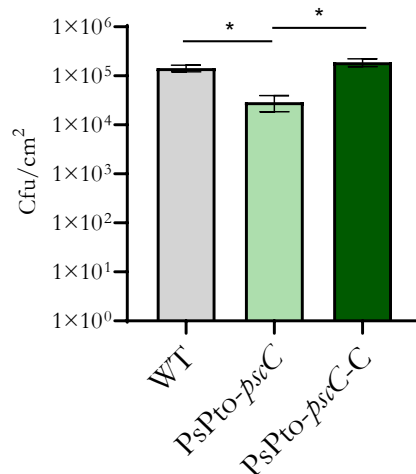


**Figure S9. Virulence assay of PsPto WT, PsPto-*pscC* mutant, and the complemented strain (PsPto-*pscC-C*) in tomato plants.** Shown are means and standard errors of bacterial populations at 6 days postinoculation after spray-inoculation ( $10^8$  cfu·ml<sup>-1</sup>). Experiments were conducted in two independent biological replicates with three technical replicates each. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ).

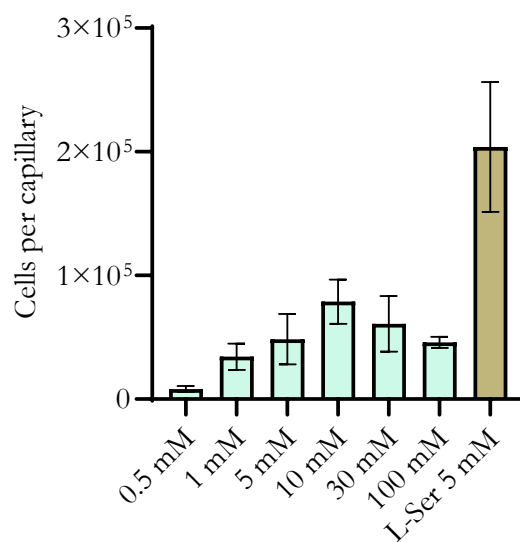


**Figure S10. Chemotaxis capillary assays of Dd3937 towards succinate at different concentrations.** L-Ser was used as a positive control at 5 mM concentration. The data was corrected with the number of cells that swam into buffer-containing capillaries. Shown are means and standard errors from three independent experiments conducted in triplicate.

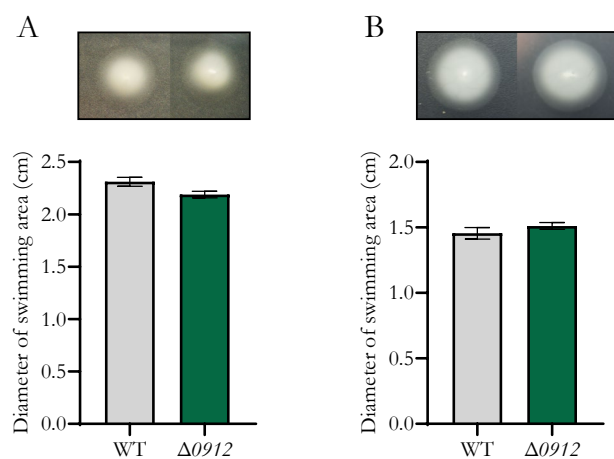


**Figure S11. Swimming motility assay of  $\Delta 0912$  mutant strain in (A) KB and (B) M9 10 mM citrate.** Shown are means and standard errors of three independent experiments with four technical replicates each.



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## **Annexes II**



# Chemosensory systems interact to shape relevant traits for bacterial plant pathogenesis

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**ABSTRACT** Chemosensory systems allow bacteria to respond and adapt to environmental conditions. Many bacteria contain more than one chemosensory system, but knowledge of their specific roles in regulating different functions remains scarce. Here, we address this issue by analyzing the function of the F6, F8, and alternative (non-motility) cellular functions (ACF) chemosensory systems of the model plant pathogen *Pseudomonas syringae* pv. tomato. In this work, we assign PsPto chemoreceptors to each chemosensory system, and we visualize for the first time the F6 and F8 chemosensory systems of PsPto using cryo-electron tomography. We confirm that chemotaxis and swimming motility are controlled by the F6 system, and we demonstrate how different components from the F8 and ACF systems also modulate swimming motility. We also determine how the kinase and response regulators from the F6 and F8 chemosensory systems do not work together in the regulation of biofilm, whereas both components from the ACF system contribute together to regulate these traits. Furthermore, we show how the F6, F8, and ACF kinases interact with the ACF response regulator WspR, supporting crosstalk among chemosensory systems. Finally, we reveal how all chemosensory systems play a role in regulating virulence.

**IMPORTANCE** Chemoperception through chemosensory systems is an essential feature for bacterial survival, as it allows bacterial interaction with its surrounding environment. In the case of plant pathogens, it is especially relevant to enter the host and achieve full virulence. Multiple chemosensory systems allow bacteria to display a wider plasticity in their response to external signals. Here, we perform a deep characterization of the F6, F8, and alternative (non-motility) cellular functions chemosensory systems in the model plant pathogen *Pseudomonas syringae* pv. tomato DC3000. These chemosensory systems regulate key virulence-related traits, like motility and biofilm formation. Furthermore, we unveil an unexpected crosstalk among these chemosensory systems at the level of the interaction between kinases and response regulators. This work shows novel results that contribute to the knowledge of chemosensory systems and their role in functions alternative to chemotaxis.

**KEYWORDS** chemoperception, *Pseudomonas syringae*, motility, biofilm, crosstalk, virulence

Signal transduction systems allow bacteria to perceive environmental stimuli and orchestrate an appropriate response to improve survival and growth. Among these systems are the chemosensory systems, specialized two-component signal transduction systems that arise from an evolutionary variation of the former to acquire an adaptative and amplified response (1). The best-known chemosensory system is the chemotaxis system, which allows the control of motility toward attractants or away from repellents by modulating the motility patterns (2). However, these systems appear to be involved

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not only in the control of flagellar function but also in the regulation of other cellular functions such as type IV pilus-based motility, biofilm formation, or the control of second messenger levels (3–5).

The core of a chemosensory pathway is formed by a complex composed of one or more chemoreceptors (CR) or methyl-accepting chemotaxis proteins, the CheA histidine kinase, and the CheW adaptor protein (6). In the canonical pathway, signal binding to the CR ligand-binding domain (LBD) creates a molecular stimulus that modulates CheA autophosphorylation, which in turn alters the transphosphorylation of the response regulator CheY. In the case of a chemotaxis pathway, phosphorylated CheY (CheY-P) binds to the flagellar motor, controlling the direction of flagellar rotation and ultimately bacterial motility. The adaptability of the system, allowing the response to stimuli over a broad concentration range, comprises the activity of the CheR methyltransferase and the CheB methylesterase, which, respectively, add and remove methyl groups from CRs modulating their affinity to the chemical stimulus. The pathway also features the CheZ phosphatase, which dephosphorylates CheY, thereby halting the signaling process (7).

The number of chemoreceptors present in a bacterium and their ligand-binding specificity are associated with its ability to perceive and respond to environmental changes. Diverse chemosensory systems have been found in different bacteria, showing variations in their components (8). Moreover, it is not unusual to find genes encoding different chemosensory transducer elements within a given bacterial genome, which suggests a versatile response capacity through different specialized pathways (9).

Chemosensory systems are classified into three main functional groups: those that evolve to control flagellar motility (F), type IV pilus-based motility (Tfp), and those involved in alternative (non-motility) cellular functions (ACF) like the control of levels of second messengers (10). The F group is the most diverse and contains 17 classes (F1–F17), while the other two groups contain a single system class.

Proteins of chemosensory systems (CRs and signal transducer elements) assemble into macromolecular complexes known as chemosensory arrays (11). Clustering enables chemosensory complexes to react collectively, thereby enhancing their sensitivity to fluctuations in environmental conditions (12, 13).

Different CRs present particular LBDs, which are the hallmark of their specificity. Genomic analysis of CR sequences predicts nearly 100 distinct LBD types (14, 15). However, the cytoplasmic signaling domain is less diverse and can be classified into seven classes based on the number and organization of the characteristic seven-residue heptads (H) present in this domain. This feature allows the classification of CRs based on the length of their signaling domain. The physical length of receptors determines whether they can be incorporated into the same chemosensory array (16, 17). This means that CRs with different ligand-binding specificities but with the same number of heptads can arrange together facilitating signal integration (18).

In bacteria with multiple chemosensory systems, the spatial segregation of arrays formed by each system, comprising different CRs, would be advantageous for preventing interference among different functions (19). However, crosstalk between different chemosensory systems has been reported (20–23). Therefore, it remains an open question if and how different chemosensory systems are structurally and functionally separated in a given species (11).

Phytopathogenic bacteria present a high number of CRs and often have multiple chemosensory systems. While 90% of phytopathogenic bacteria possess chemotaxis-related genes (14), such genes are only found in 47% of all bacteria (24). There is abundant experimental evidence that highlights the importance of chemotaxis for efficient plant colonization (25–36).

*Pseudomonas syringae* pv. tomato DC3000 (PsPto) is the causal agent of bacterial speck in tomatoes and is considered an aggressive pathogen once inside the plant (37, 38). However, it is a weak epiphyte compared to other *P. syringae* strains (39), suggesting that it needs to rapidly perceive and respond to environmental and plant signals to ensure entry into the plant apoplast (40, 41). Stomata are the main entry points of

PsPto to the apoplast, and it has been shown that PsPto is able to actively move toward open stomata (42). Biofilm formation is also involved in adaptation to environmental stress such as that generated during the interaction between PsPto with plants (43). Pathogenicity has been shown to be associated with increased motility and decreased cell aggregation (44). Therefore, these traits must be tightly regulated to adapt to the host environment and ensure efficient plant colonization.

PsPto contains four main chemosensory systems in independent gene clusters: cluster I (F6), cluster II (F8), cluster III (ACF), and cluster IV (Tfp). The 49 CRs that interact with these systems are scattered across the genome. Previous results determined that cluster I is responsible for chemotaxis (33), as a defective *cheA2* mutant lost chemoattraction to several compounds. Moreover, this mutant also displayed an altered phenotype in the levels of the second messenger cyclic diguanosine monophosphate (c-di-GMP), suggesting crosstalk between chemosensory systems (33). In addition, it has been shown that a PsPto *cheA2* mutant is impaired in virulence in tomato plants (45).

Although previous studies on PsPto and *P. syringae* pv. *tabaci* have analyzed phenotypes associated with mutations in the F6 and F8 chemosensory systems in these bacteria (45, 46), the way the different chemotaxis pathways connect and interplay to facilitate adaptation to plant host has not yet been elucidated.

In this work, we propose an assignment of each of the 49 CRs to any of the main four chemosensory systems found in this bacterium. We show, for the first time, the visualization of the primary chemosensory arrays in this bacterium and analyze the function, crosstalk, and interaction among components of the main PsPto chemosensory systems (F6, F8, and ACF) in relation to pathogenicity traits.

## MATERIALS AND METHODS

### Bacterial strains, growth conditions, and plasmids

Bacteria and plasmids used in this study are listed in Table S1. PsPto derivatives were grown at 28°C in King's B (KB) medium (47). *Escherichia coli* derivatives were grown at 37°C in lysogeny broth (LB) (48). When appropriate, antibiotics were added to the medium at the following concentrations: rifampicin 25 µg/mL, kanamycin 50 µg/mL, and ampicillin 100 µg/mL.

### Construction of deletion mutant strains and complementation strains

Generation of deletion mutants was carried out by double crossover and sucrose counter-selection using pK18mobsac (49). Complementation strains were obtained by expressing the corresponding gene in each mutant background using the broad-host-range plasmid pBBR1MCS-2 (50). The  $\Delta cheA2$  was complemented using the pME6010::CheA2 plasmid (45). A detailed description can be found in Text S1.

### Prediction of chemoreceptor arrays' height

To obtain the classification of chemosensory proteins and chemoreceptors, we used the MiST4.0 (51) database. We retrieved the protein sequence in fasta format and annotations of transmembrane regions. To build the atomic models from the sequences, we used ColabFold v1.5.2 (52) with default parameters. This version of ColabFold uses AlphaFold 2 (53) with MMseqs2 (54) in batch mode. The atomic structure was analyzed using VMD 1.9.4a57 (55) to measure the size of the receptor from the tip of the receptor to the amino acid from the transmembrane region closest to the cytoplasm.

### Cryo-electron tomography

For sample preparation, PsPto strains were grown overnight in KB liquid medium at 28°C. Cells were washed twice with M9 minimal media supplemented with 10 mM citrate and concentrated by centrifugation at  $3,000 \times g$  for 5 min. Protein A-treated

10 nm colloidal gold solution (Cell Microscopy Core, Utrecht University, Utrecht, The Netherlands) was added to the samples at a dilution of 1:20 and mixed gently before plunge-freezing. Subsequently, 3  $\mu$ L of the sample was applied to a freshly glow-discharged, carbon-coated copper grid R2/2, 200 mesh grids (Quantifoil Micro Tools GmbH, Großlöbichau, Germany). Plunge-freezing was performed on an EM GP plunge freezer (Leica Microsystems, Wetzlar, Germany) in liquid ethane under the following conditions: pre-blotting time, 30 seconds; blotting time, 1 second; humidity, 85%; and chamber temperature, 20°C.

Data were acquired using a TITAN Krios 300 kV cryo-transmission electron microscope (Thermo Fisher Scientific, Hillsboro, OR, USA), and images were recorded with a K3 direct electron detector (Bioquantum, Gatan, Pleasanton, CA, USA) with a slit width of 20 eV. Images were acquired at a magnification of  $\times 19,500$ , which corresponds to a pixel size of 4.411 Å. Tilt series were collected using SerialEM 4.1 (56) with a bidirectional tilt scheme from  $-60^\circ$  to  $60^\circ$ , starting from  $0^\circ$  with  $2^\circ$  increments. The cumulative exposure was set to 157 e-/Å and the defocus to  $-6 \mu\text{m}$ . From each variant, 48–50 tilt series were collected, and three-dimensional reconstructions were generated using the IMOD software package (57, 58). The tomograms were screened for the presence of visible chemotaxis arrays. Only tomograms with chemotaxis arrays in side view were further processed.

### Measurement of chemotaxis arrays from cryo-tomograms

The measurement of the average array density profiles was calculated automatically using the “sideview-profile-average” script (59). Briefly, model points were generated in tomograms along the inner membrane and chemotaxis arrays. Based on these model points, the script calculates the average pixel value perpendicular to the model points in the same plane to create JSON files with the average pixel profile. The profiles were visualized in the ObservableHQ notebook to measure the distance between the density dips of the inner membrane and the CheA/CheW plane (Fig. S1).

### Capillary chemotaxis assays

Cultures grown overnight were diluted to an  $\text{OD}_{600}$  of 0.05 in KB medium and grown at 28°C with orbital shaking. At the early stationary phase of growth, cultures were centrifuged at  $1,740 \times g$  for 5 min, and the resulting pellet was washed twice with 10 mM HEPES (pH 7.0). Cells were resuspended in HEPES and adjusted to an  $\text{OD}_{600}$  of 0.25. Next, 230  $\mu$ L of cells were placed into each well of a 96-well plate. One-microliter capillaries were filled with the compound to be tested, immersed into the bacterial suspension, and incubated for 30 min. Capillaries were removed from the bacterial suspension and rinsed with sterile water, and the content was expelled into 1 mL of nutrient broth (NB) medium (1 g yeast extract, 2 g beef extract, 5 g NaCl, and 5 g Bacto Peptone [per liter]). Serial dilutions were plated onto NB medium with the appropriate antibiotics, and the number of colony-forming units (CFU) was determined. In all cases, data were corrected by subtracting the number of cells that swam into buffer-containing capillaries.

### Swimming motility assays

PsPto strains were grown on KB agar plates at 28°C for 48 hours. Individual colonies were picked and inoculated into soft KB agar plates, M9 citrate (60) agar plates, or HRP (61) medium agar plates (0.3%, wt/vol), a medium that mimics plant apoplast. Soft agar plates were incubated at 28°C in a humid environment under dark conditions. The diameter of the swimming halo was measured at different time points.

### Attachment assays

Attachment assays were performed as described previously by Chakravarthy et al. (44), with a modified MG liquid medium, MGA medium (54 mM mannitol, 3.6 mM  $\text{KH}_2\text{PO}_4$ ,

23 mM NaCl, 0.8 mM MgSO<sub>4</sub>, and 18 mM NH<sub>4</sub>Cl [pH 7.0]). A detailed description can be found in Text S1.

### Colony morphology and Congo red binding assays

Overnight cultures grown in KB liquid medium at 28°C were washed with KB medium and diluted to an OD<sub>600</sub> of 0.05. Twenty microliters of diluted culture was inoculated either in KB agar plates (1.5%, wt/vol) supplemented with Congo red (40 µg/mL) and Coomassie brilliant blue (20 µg/mL) or M9 citrate agar plates (1.5%, wt/vol) supplemented with Congo red (40 µg/mL). Pictures were taken with ZEISS Axio Zoom.V16 stereoscope at different time points.

### Flocculation assays

PsPto strains were grown overnight at 28°C in KB medium and left statically at RT for at least 24 hours. Pictures were taken when flocculation was observed.

### Bacterial two-hybrid assays

Bacterial strains and plasmids were purchased from Euromedex (France). The reporter strain *E. coli* DHM10 (cya<sup>-</sup>) was co-transformed with two-hybrid plasmids. Clones were selected on LB agar plates supplemented with ampicillin (100 µg/mL) and kanamycin (50 µg/mL). Three clones of each co-transformation were inoculated in LB medium supplemented with kanamycin (50 µg/mL) and ampicillin (100 µg/mL) and grown at 37°C and 200 rpm for at least 4 hours. Two microliters of bacterial culture was spotted on LB plates supplemented with kanamycin (50 µg/mL), ampicillin (100 µg/mL), 0.5 mM IPTG, and X-gal (40 µg/mL). Plates were incubated at RT or 28°C until colonies turned blue. A detailed description can be found in Text S1.

### Virulence assays

PsPto strains were grown at 28°C for 24 hours on KB agar in the dark. Cells were resuspended in 10 mM MgCl<sub>2</sub> and diluted to 10<sup>8</sup> CFU/mL. For spray inoculation assays, 3-week-old tomato plants (*Solanum lycopersicum* cv. Moneymaker) were sprayed with a suspension containing 10<sup>8</sup> CFU/mL, and Silwet L-77 was added at a final concentration of 0.02% (vol/vol). Plants were incubated in a growth chamber at 25°C and 60% RH with a daily light period of 12 hours. Six days post-inoculation (dpi), the leaf symptoms were recorded, and bacterial populations from three plants were measured by sampling five 1-cm-diameter leaf disks per plant. A detailed description can be found in Text S1.

For syringe-infiltration assays, cells were resuspended in 10 mM MgCl<sub>2</sub> and diluted to 3 × 10<sup>4</sup> CFU/mL. Tomato leaves were pierced with a needle on the adaxial side of the leaves, and 100 µL of bacterial suspension was syringe-infiltrated through the wound. Plants were incubated in a growth chamber at 25°C and 60% RH with a daily period of 12 hours. Three days after inoculation, the leaf symptoms were recorded, and bacterial populations were determined.

### Statistical analysis

All statistical analyses were performed using the GraphPad Prism software. When samples followed a normal distribution, a *t*-test or one-way analysis of variance (ANOVA) was performed. When ANOVA was used, multiple comparisons were performed using Fisher's least significant difference *post hoc* test. When samples did not follow a normal distribution, a Mann-Whitney test or a Kruskal-Wallis test was used. Multiple comparisons were performed using Dunn's test.

## RESULTS

### Assigning chemoreceptors to chemosensory systems in PsPto

Most PsPto chemotaxis genes are arranged in four gene clusters (clusters I–IV) called chemosensory systems. Additional chemotaxis genes are located across the genome

with no particular organization. PsPto main clusters were initially numbered according to their homology with other *Pseudomonas* species (*Pseudomonas aeruginosa* PAO1 and *Pseudomonas putida* KT2440) (62) on the basis of their genomic organization. Genes in the clusters were annotated before the cluster numbering, and therefore the numbering of genes differs from the numbering of the chemosensory system they belong to (Fig. 1).

All chemotaxis and chemoreceptor genes from PsPto were retrieved using the MiST4.0 database (51). MiST4.0 associates chemotaxis genes with specific chemosensory categories depending on gene orthology, protein structure, and experimental evidence in related bacteria (63). MiST4.0 predicts that PsPto has 49 chemoreceptors (41 from the 40H class, 1 from the 36H class, 1 from the 34H class, and 6 from the 24H class). The four PsPto chemosensory systems are categorized as F6, F8, ACF, and Tfp (Fig. 1).

In bacteria with a single chemosensory system, the 24H receptor class has been assigned to work with F1, F3, F5, F6, and F7 systems (64). Since only the F6 system is present in PsPto, we assign all six 24H receptors to the F6 system (cluster I). The F6 system is known to also work with 40H receptors (64); therefore, we assign all 40H chemoreceptors to the F6 system, except for two chemoreceptors, *PSPTO\_1493* and *PSPTO\_5031*. *PSPTO\_1493* exhibits synteny and shares 69.2% identity with the *wspA* gene of PAO1, with a genomic location in the ACF chemotaxis cluster. *PSPTO\_5031* exhibits synteny and shares 77.4% identity with the *pilJ* gene of PAO1, encoded in the Tfp chemotaxis cluster. Therefore, we assign *PSPTO\_1493* and *PSPTO\_5031* to the ACF and Tfp systems, respectively (Fig. 1; Table S2). Moreover, experimental data are available on the homologous chemoreceptors of PAO1 showing their function in association with these classes of chemosensory systems (65, 66). *PSPTO\_0912* is the only receptor belonging to the 34H class, which is a minor class of chemoreceptors. *PSPTO\_0912* is

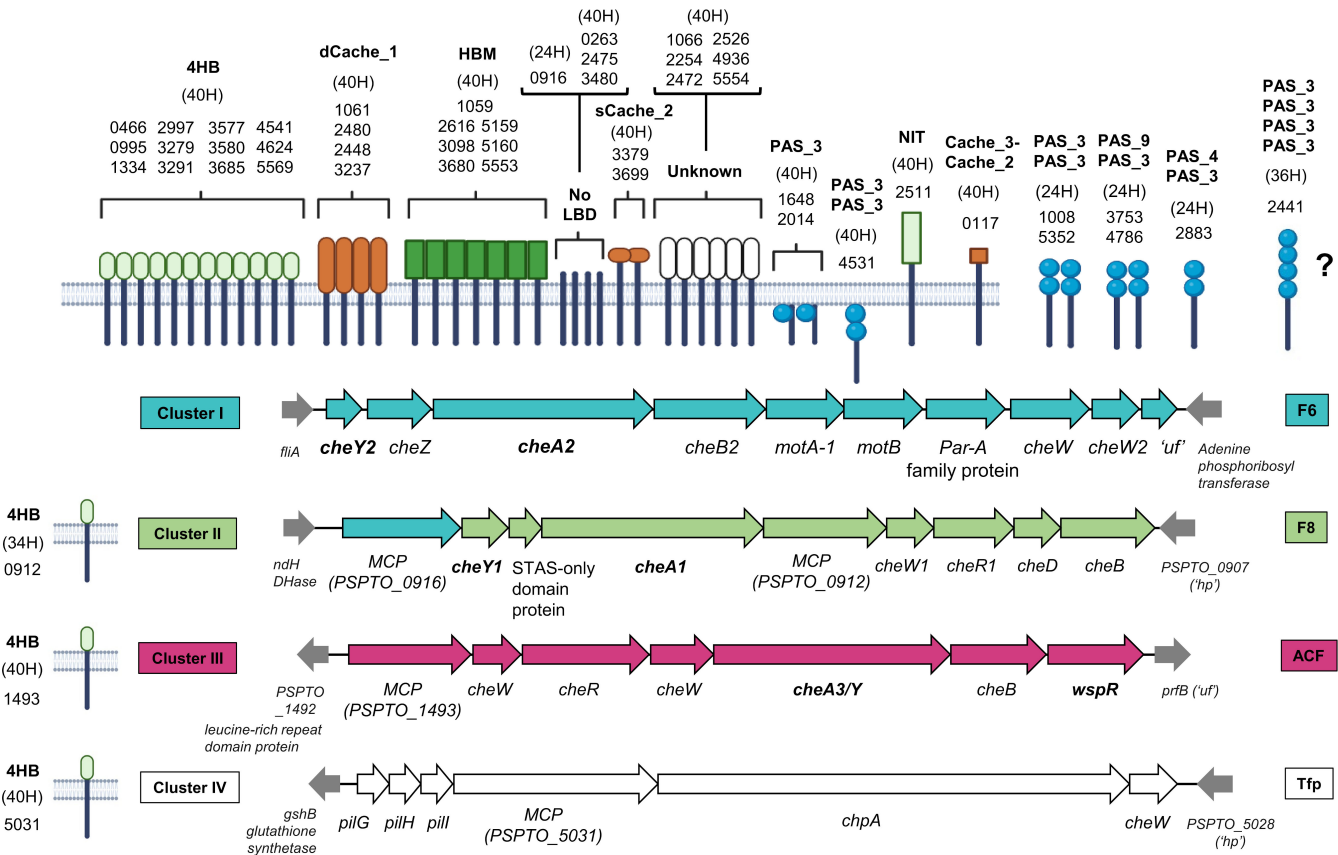


**FIG 1** Chemoreceptors and chemosensory systems of *Pseudomonas syringae* pv. tomato DC3000. Chemoreceptors are grouped by LBD topology and chemosensory system association. Chemoreceptor class is indicated by the number of heptads (H). NCBI accession numbers (PSPTO\_XXXX) are indicated below the class type. Nomenclature for the LBD types is the same as in Sanchis-López et al. (24). Genes mutated in this work are shown in boldface type.

encoded in the F8 gene cluster (cluster II), suggesting it functions in association with this system. Moreover, the assignment of 34H chemoreceptors to the F8 system has been previously proposed in other organisms (59, 67) (Fig. 1; Table S2). [PSPTO\\_2441](#) is the only chemoreceptor belonging to the 36H class, which has been mostly assigned to the F1, F3, F7, and Tfp classes (64). From these systems, only Tfp is present in PsPto; therefore, we could assign [PSPTO\\_2441](#) to the Tfp class (Fig. 1; Table S2). However, [PSPTO\\_2441](#) is a cytoplasmic CR harboring four PAS domains. Most CRs with PAS domains have been assigned to the F6 system; therefore, a definitive assignment cannot be performed for this CR.

### Cryo-electron tomography reveals two distinct chemotaxis arrays in PsPto

Of all chemosensory systems found in PsPto, only the F6 type has been successfully imaged before in other bacteria using cryo-electron microscopy (59). To our knowledge, F8, ACF, and Tfp systems have not been previously imaged. In the case of ACF and Tfp systems, it is still unknown if they assemble clusters large enough to be visible with cryo-electron microscopy. Thus, we focused on building structural models for the F6 and F8 systems using two chemoreceptor sequences from classes 40H ([PSPTO\\_2480](#)) and 34H ([PSPTO\\_0912](#)). In the case of the 34H chemoreceptor, we measure 20 nm from the amino acid I215 at the base of the transmembrane region to the G380 at the tip of the receptor. In the case of the 40H chemoreceptor, we measure 23 nm from the amino acid M301 to the G488 at the tip of the chemoreceptor. Thus, we predict that the F8 system will have a height of 20 nm and the F6 system a height of 23 nm.

We used cryo-electron tomography to visualize and characterize the chemoreceptor arrays in PsPto (Fig. 2). We were able to visualize and identify two distinct arrays in wild-type (WT) cells measuring 20 and 23 nm in height. This is consistent with our predicted measurements, thus identifying the arrays as F8 and F6 systems, respectively. In *E. coli*, the absence of CheA kinase impedes the formation of the chemotaxis array. Since CheA1 is predicted to take part in the F8 array and CheA2 in the F6 array, we

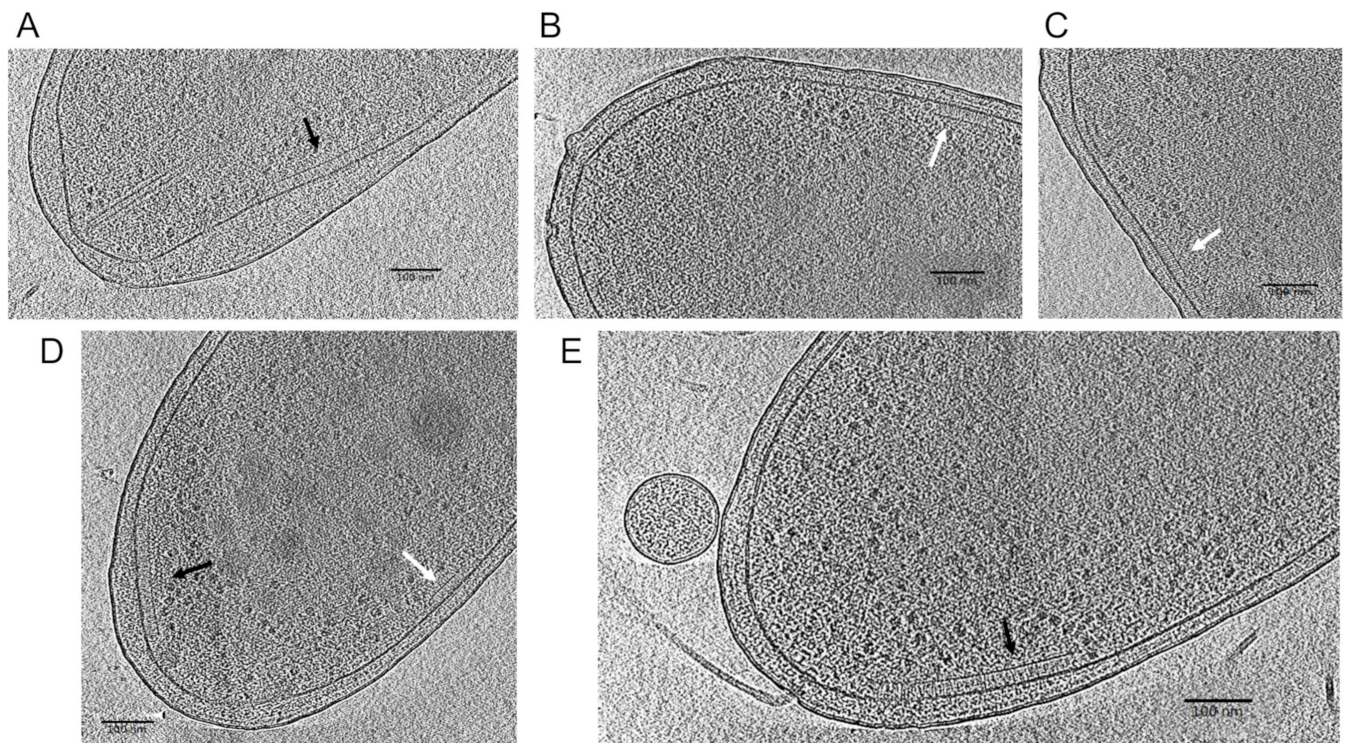


**FIG 2** Cryo-electron microscopy of WT,  $\Delta cheA1$ , and  $\Delta cheA2$  cells from PsPto. Black arrows point to chemosensory arrays of type F6, and white arrows point to chemosensory arrays of type F8. (A) WT with F6 array. (B) WT with F8 array. (C)  $\Delta cheA2$  with F8 array. (D)  $\Delta cheA2$  with F6 and F8 arrays. (E)  $\Delta cheA1$  with F6 array.

also imaged cells from  $\Delta cheA1$  and  $\Delta cheA2$  strains to confirm which kinase forms each array.  $\Delta cheA1$  cells only showed F6 arrays, which indicates that CheA1 participates in the formation of F8 arrays. Surprisingly,  $\Delta cheA2$  showed both F6 and F8 arrays (Fig. 2; Table S3).

### Chemotaxis is impaired in $\Delta cheA2$ but not in $\Delta cheA1$ and $\Delta cheA3$ PsPto mutant strains

To unveil the role of the different PsPto chemosensory systems in the control of flagellar motility, we performed capillary-based chemotaxis assays against casamino acids, which are strong chemoattractants for *Pseudomonas*. We tested this phenotype in the  $\Delta cheA1$ ,  $\Delta cheA2$ , and  $\Delta cheA3$  mutants altered in the kinase of each chemosensory system. Results showed that only the F6 system is involved in the control of chemotaxis in PsPto (Fig. 3). These results are in line with the previously reported involvement of this system in amino acid perception (33).

### Chemosensory systems regulate swimming motility

To assess the role of the PsPto chemosensory systems in chemotaxis-related motility, we performed soft agar swimming assays in strains lacking either *CheA* or the corresponding response regulator in each chemosensory system (Fig. 4A). All strains lacking *CheA* showed swimming defects, but the deletion of *cheA2* completely abolished motility in all media tested.  $\Delta cheY2$  displayed the same swimming phenotype as  $\Delta cheA2$  in all media tested, and  $\Delta wspR$  displayed a similar phenotype as  $\Delta cheA1$  and  $\Delta cheA3$ , showing a decreased swimming motility. However,  $\Delta cheY1$  showed a similar motility phenotype to the WT strain in all media tested. Swimming was partially restored in the  $\Delta cheA2$ -complemented strain and totally restored in the  $\Delta cheY2$ -complemented strain (Fig. S2). Swimming complementation of  $\Delta cheA1$ ,  $\Delta cheA3$ , and  $\Delta wspR$  strains was not observed (data not shown).

CheY-type response regulators bind to the flagellar motor protein FliM to modulate flagellar rotation (68). Based on the two opposite motility phenotypes observed in the two PsPto  $\Delta cheY$  mutants, we carried out bacterial adenylate-cyclase two-hybrid (BACTH) assays to test the potential interactions between PsPto FliM and both CheY proteins. Results showed that CheY2 interacts with FliM, while no binding was detected between CheY1 and FliM (Fig. 4B).

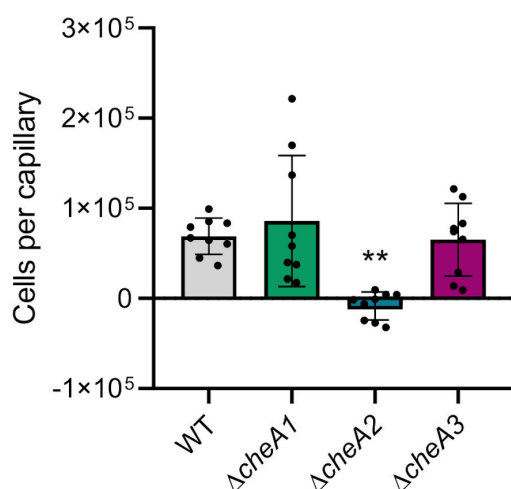


**FIG 3** Chemotaxis capillary assays against 0.1 mM casamino acids. The data have been corrected with the number of cells that swam into buffer-containing capillaries. Bars represent the means of three independent experiments with three technical replicates each. Values that are significantly different are indicated by asterisks (\*\* $P < 0.01$ ).

## Chemosensory systems contribute to biofilm-related phenotypes

Attachment assays were performed using the crystal violet method described previously (44) (Fig. 5A).  $\Delta cheA1$ ,  $\Delta cheA2$ , and  $\Delta wspR$  showed a significant increase in attachment when compared to the WT. Contrarily,  $\Delta cheY1$  and  $\Delta cheY2$  attached significantly less than the WT strain. A  $cheA3$  deletion did not cause any attachment alteration in this assay.

We decided to analyze the Congo red uptake ability, which is related to exopolysaccharide (EPS) production and indirectly to biofilm formation (69) (Fig. 5B). First, we observed that  $\Delta cheA1$ ,  $\Delta cheA2$ ,  $\Delta cheA3$ , and  $\Delta wspR$  mutants presented a higher Congo red uptake ability than the WT. In contrast,  $\Delta cheY1$  and  $\Delta cheY2$  showed Congo red uptake similar to that of the WT strain. Flocculation phenotype in liquid media is also related to EPS production, and we also observed that liquid cultures of  $\Delta cheA1$ ,  $\Delta cheA2$ ,  $\Delta cheA3$ , and  $\Delta wspR$  flocculated overnight when left static at room temperature, whereas the WT flocculated partially, and  $\Delta cheY1$  and  $\Delta cheY2$  remained homogeneously distributed across the liquid media (Fig. 5C). We also analyzed the colony morphology and texture in rich medium, which have also been related to the biofilm formation ability (70) (Fig. S3). We could observe how the inner colony areas of  $\Delta cheA1$ ,  $\Delta cheA2$ ,  $\Delta cheA3$ , and  $\Delta wspR$  exhibited more pronounced wrinkling as compared to the WT. In contrast,  $\Delta cheY1$  and  $\Delta cheY2$  displayed a smooth colony morphology.

## Bacterial two-hybrid assays reveal unusual interaction partners

The above-described results point to a possible crosstalk between the three chemosensory systems analyzed in this work (F6, F8, and ACF). Canonically, we would expect

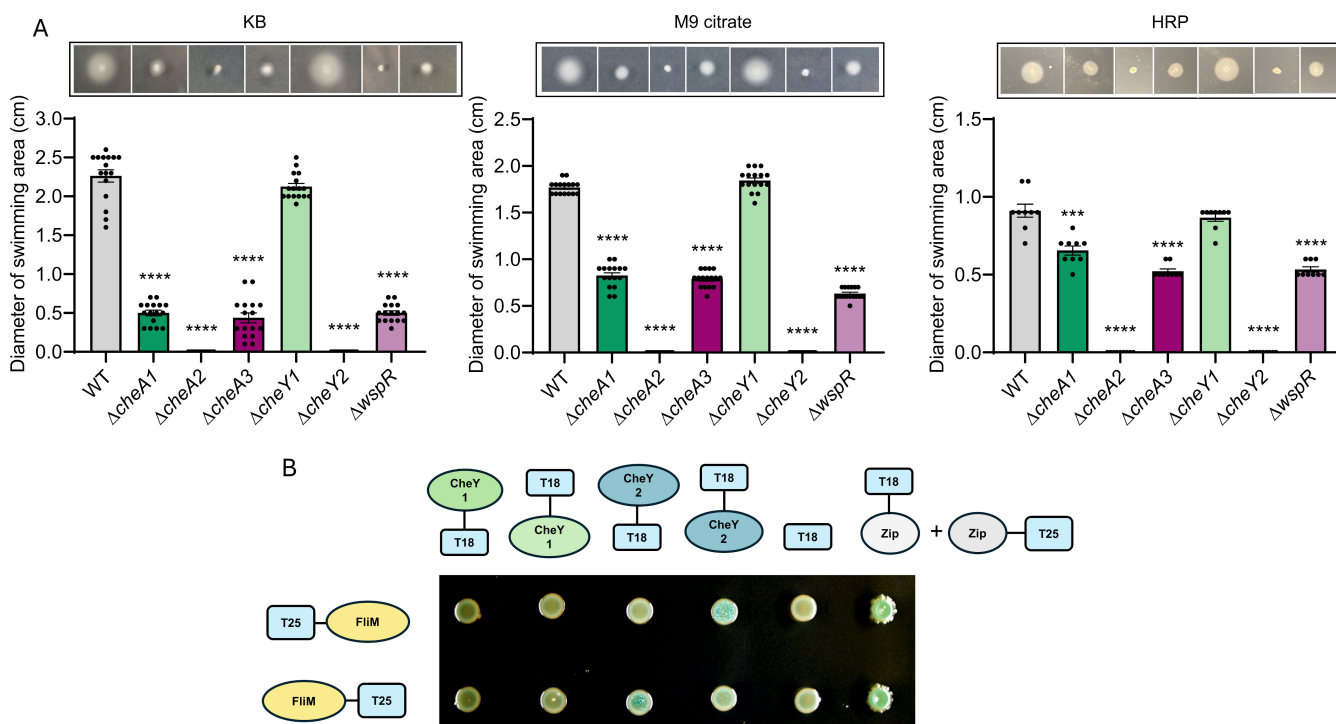


**FIG 4** Swimming motility assays and protein interactions between FlIM and CheY response regulators. (A) Swimming motility assays in KB, M9 citrate, or HRP medium agar plates (0.3%, wt/vol). Bars represent the means of four independent experiments with four technical replicates each. Values that are significantly different are indicated by asterisks ( $***P < 0.001$  and  $****P < 0.0001$ ). (B) Bacterial adenylate cyclase two-hybrid assays between FlIM and the CheY1 and CheY2 proteins from PsPto. Experiments were conducted in triplicate with three technical replicates each. For better visualization, only one replicate is shown for each interaction. A schematic representation of proteins cloned at the N-terminal or C-terminal of the T25 or T18 adenylate cyclase fragment is presented. FlIM was tested against the T18 fragment of the adenylate cyclase as a negative control. Interaction between the leucine zipper of GCN4 in frame with the T25 and T18 fragments of the adenylate cyclase was used as a positive control.

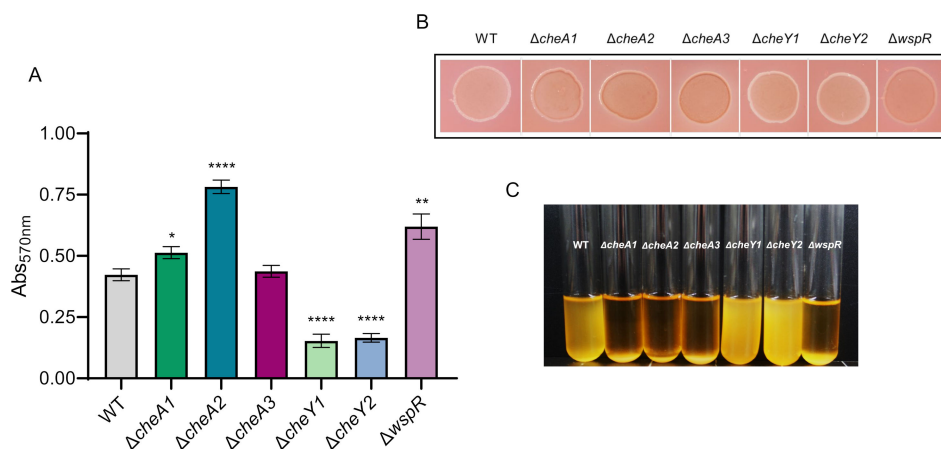


**FIG 5** Biofilm-related traits. (A) Crystal violet attachment assay. Bars represent the means of three independent experiments with six technical replicates each. Values that are significantly different are indicated by asterisks (\* $P < 0.05$ ; \*\* $P < 0.01$ ; and \*\*\*\* $P < 0.0001$ ). (B) Representative image of the Congo red uptake by colonies grown on M9 citrate plates. Experiments were conducted in triplicate with six technical replicates each. (C) Flocculation assays of the WT and mutant strains. Overnight cultures were left to flocculate at room temperature overnight. Three independent experiments were conducted with three technical replicates each. For better visualization, only one replicate is shown for each strain. White arrow points to the sedimentation area.

the kinase CheA2 to interact with the response regulator CheY2 (F6 system), CheA1 to interact with CheY1 (F8 system), and CheA3 to interact with WspR (ACF system). BACTH assays were performed to check interactions among the three kinases and the three response regulators. Results showed that, as expected, CheA2 and CheA3 interact with their canonical response regulators (Fig. 6). Surprisingly, we were not able to detect the interaction between CheA1 and CheY1 response regulator. Moreover, a clear interaction was observed in the case of both CheA1 and CheA2 with the response regulator WspR (Fig. 6).

## Chemosensory systems are necessary for an optimal plant entry to incite infection

We tested the ability of PsPto chemosensory mutant strains to infect tomato plants. We used different inoculation methods, the spray-inoculation approach, which mimics natural entry conditions that bacteria face in the phyllosphere, and the infiltration method, in which the pathogen is placed inside the plant. Bacterial populations of mutant strains were reduced compared to that of the WT strain when cells were spray-inoculated. However, this difference was abolished when the strains were inoculated by infiltration (Fig. 7). Nevertheless, we could observe how syringe-inoculated tomato leaves with  $\Delta cheA3$  and  $\Delta wspR$  mutant strains displayed a decreased necrotic area than the WT strain (Fig. S4). These findings demonstrate how all three chemosensory systems play a key role during the early stages of the infection of tomato plants. Furthermore, the ACF system also seems to be involved in symptom development.

## DISCUSSION

Many bacteria have evolved more than one chemosensory system (10), which is arranged independently, and it has been proposed that this cluster-based scaffolding allows the spatial separation of chemotaxis pathways putatively involved in specific cellular functions (19).

PsPto has four main chemosensory clusters, namely, F6, F8, ACF, and Tfp systems, but many questions remain to be answered regarding the specific functions they control. Here, we have assigned all 49 chemoreceptors of PsPto to one of the four

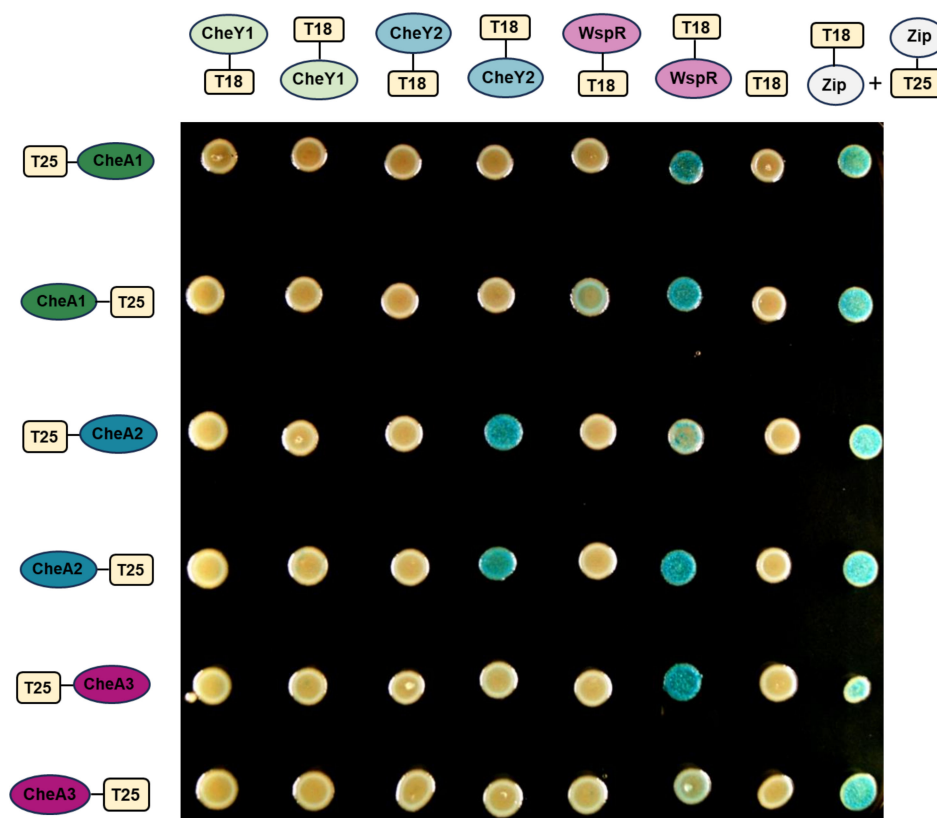


**FIG 6** Bacterial adenylate cyclase two-hybrid assays between CheAs and response regulators from PsPto. Experiments were conducted in triplicate with three technical replicates each. For better visualization, only one replicate is shown for each interaction. A schematic representation of proteins cloned at N-terminal or C-terminal of the T25 or T18 adenylate cyclase fragment is presented. CheA1, CheA2, and CheA3 were tested against the T18 fragment of the adenylate cyclase as a negative control. Interaction between the leucine zipper of GCN4 in frame with the T25 and T18 fragments of the adenylate cyclase was used as a positive control.

main chemosensory clusters. The assignment is based solely on a deductive process based on previous data and assignments in other organisms (64, 71). Therefore, further experimental data will be necessary to confirm or refute our assignment.

Most of the 40H chemoreceptors have been assigned to the F6 system. However, the 40H chemoreceptors [PSPTO\\_1493](#) and [PSPTO\\_5031](#) are proposed to be assigned to the ACF and Tfp systems, respectively. This assignment is based on both genomic context and experimental data on homologous chemoreceptors of PAO1 (65, 66). Regarding [PSPTO\\_0912](#), the only 34H class chemoreceptor of PsPto, we proposed its assignment to the F8 system based on its genomic location. Moreover, it is the only chemoreceptor of PsPto containing a C-terminal pentapeptide, a domain predicted to interact with CheR proteins containing a GXX insertion (72). The only CheR in PsPto that presents this feature is also encoded in the F8 cluster ([PSPTO\\_0910](#)), which supports the assignment of [PSPTO\\_0912](#) to this system. [PSPTO\\_2441](#) is the only chemoreceptor of the 36H class. As chemoreceptors of 36H and 40H tend not to cluster together (17) and experimental data support (65) the assignment of [PSPTO\\_5031](#) (40H) to the Tfp system, we could speculate that [PSPTO\\_2441](#) is working with the F6 system instead (64).

Using cryo-electron tomography, we have been able to visualize two types of chemoreceptor arrays in PsPto, which correspond to arrays of the F6 and F8 classes. Surprisingly, in the  $\Delta cheA2$  mutant background, we still observe the presence of F6 arrays. A similar result has been observed in the case of *Azospirillum brasilense* mutant strains lacking the Che1 and Che4 clusters, corresponding to F5 and F7 systems (23). In

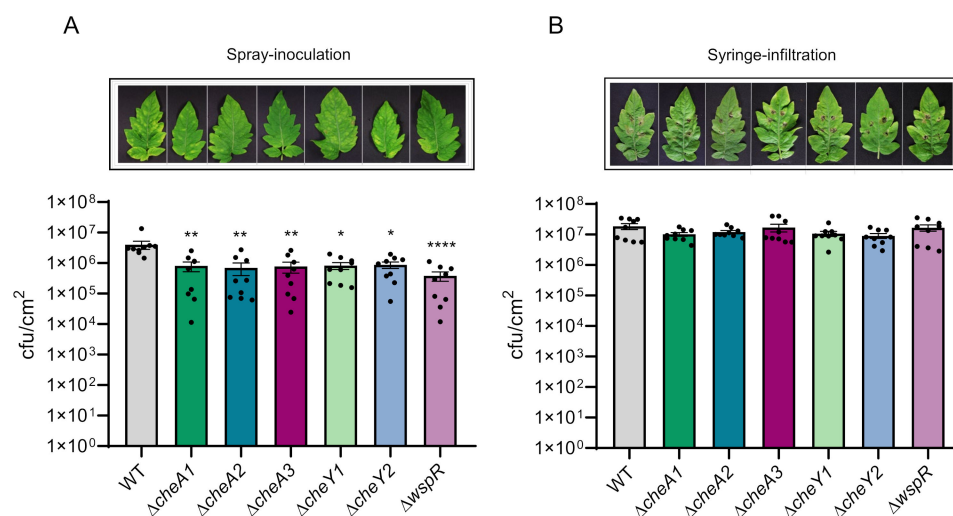


**FIG 7** Plant virulence assays with two inoculation methods. (A) Spray-inoculated virulence assays of tomato plants. Bars represent bacterial populations of strains at 6 days post-inoculation (dpi) after spray inoculation ( $1 \times 10^8$  CFU/mL) (B) Syringe infiltration virulence assay. Bars represent bacterial populations of strains at 3 dpi after syringe infiltration ( $3 \times 10^4$  CFU/mL). Shown are means and standard errors from three independent biological replicates. In each experiment, three plants were analyzed by sampling five 1-cm-diameter leaf discs per plant. Values that are significantly different are indicated by asterisks (\* $P < 0.05$ ; \*\* $P < 0.01$ ; and \*\*\*\* $P < 0.0001$ ).

this case, the authors suggest the formation of mixed baseplates in the arrays, which would support the formation of the arrays in the absence of any of the CheA proteins (23). However, we cannot discard the formation of non-functional arrays in the absence of CheA2 due to the presence of the coupling protein CheW. Actually, array formation in the absence of CheA has been experimentally shown to be possible in the case of *Vibrio cholerae* (73).

Our results confirm that in PsPto, the F6 system is required for chemotaxis and in plate swimming motility. In contrast, the F8 and ACF systems of PsPto do not seem to be involved in the regulation of chemotaxis in capillary assays, but they have a function in motility modulation in plate swimming assays. These results are consistent with previous reports in other bacteria with more than one chemosensory system, showing the function of different chemotaxis pathways regulating distinct motility parameters. In *Rhizobium leguminosarum*, the Che1 pathway has been found to be the major chemotaxis cluster controlling motility pattern, while Che2 plays a minor role (74). In the case of *A. brasilense*, Che1 controls swimming speed and has only a minor role in chemotaxis, while the Che4 system controls cell tumbling, which is essential for chemotaxis (75). Our results on the main role of CheA2 kinase in swimming motility and, therefore, of the F6 chemosensory system are in line with previous results from Clarke et al. (45). However, we also found a role for the CheA1 and CheA3 kinases in the modulation of swimming motility that was not reported in this previous work. It is worth highlighting that the authors constructed two types of mutations, disruption and deletion mutants, and the described data are based on the disruption mutant phenotypes. We have constructed clean deletion mutants and observed no defects in growth in M9 citrate medium (Fig. S5). Moreover, in *P. syringae* pv. tabaci, a pathovar closer to PsPto, the key role of the CheA2 kinase in the control of both chemotaxis and swimming motility and a minor implication of the CheA1 kinase in these phenotypes (76) have been shown. However, there are no data on the putative function of the kinase from the ACF system in this pathovar. Notably, no complementation was possible in any of the previous works showing phenotypes for CheA1 homologs. This could be due to the nature of a tight regulation of kinase functions.

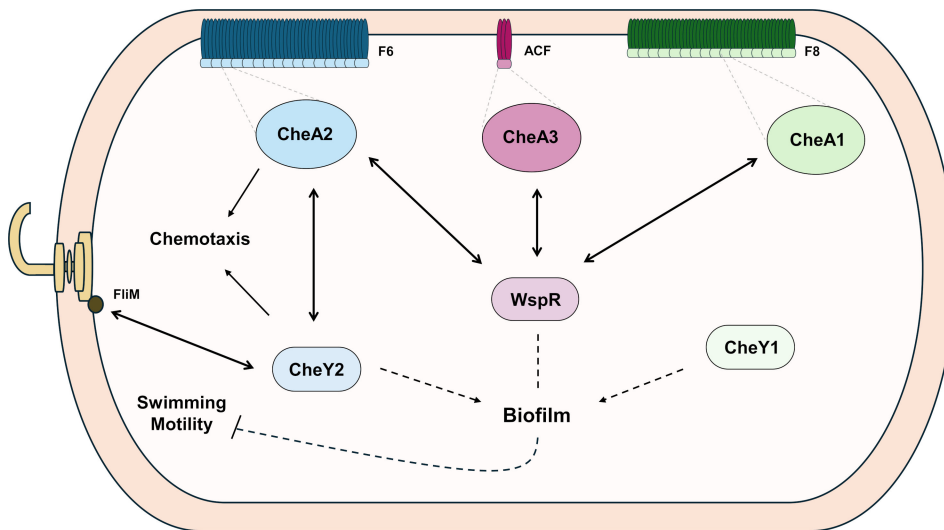


**FIG 8** Proposed model of signal transduction and crosstalk among chemosensory systems in PsPto. Double-headed arrows indicate protein interaction. Solid lines indicate regulatory functions; arrow ends indicate promotion, and dash ends indicate suppression. Dashed arrows indicate regulatory functions through unknown elements; arrow ends indicate promotion, and dash ends indicate suppression.

Our observations reveal how CheY2 is the true chemotaxis response regulator and works together with CheA2 to ensure chemotaxis and swimming (Fig. 8). However, while CheY1 does not seem to have a role in motility, the CheA1 kinase contributes to the control of this trait (Fig. 8). This reveals how CheA1 and CheY1 work independently regarding motility. In bacteria containing more than one CheY homolog (9, 77, 78), it is common that one CheY homolog is essential for the chemotaxis response while the remaining CheY homolog works as a phosphate sink. This is the case for *Sinorhizobium meliloti* and *Azorhizobium caulinodans*. It is consistent that a mutation in the essential response regulator abolishes swimming behavior, while a mutation in the phosphate sink response regulator decreases swimming performance (77, 79). However, this does not seem to be the case in PsPto, as a  $\Delta cheY1$  mutant does not show any defect in swimming motility. The dual role of two CheY homologs was also assessed in *Agrobacterium fabrum* (78). In this case, CheY1 and CheY2 from *A. fabrum* were both discovered to contribute to chemotaxis by binding FliM with different affinities, which culminated in different rates of swimming reversals, but both were shown to interact with the same CheA (78). In PsPto, we have shown the interaction between CheY2 and FliM, although the interaction between CheY1 and FliM could not be detected. This could explain the absence of a swimming phenotype in the  $\Delta cheY1$  mutant.

Biofilm formation has been primarily described as a feature under the control of ACF systems. However, several reports over the last years have shown how F systems also contribute to the regulation of biofilm formation (20–23, 80). Indeed, we have observed an increased biofilm formation in  $\Delta cheA1$  and  $\Delta cheA2$  mutant strains, which belong to the F8 and F6 systems, respectively. The highest attachment capacity (which is the first step of biofilm formation) observed in the  $\Delta cheA2$  mutant aligns with the phenotype observed in a *cheA* mutant strain of *E. coli* (81). Moreover, these observations are in line with the findings in PAO1 where the CheA kinase has been described to interact with the biofilm dispersal protein DipA phosphodiesterase involved in the control of c-di-GMP homeostasis (82).

Surprisingly, in terms of biofilm-related traits, mutants in the CheY response regulators displayed opposed phenotypes than those of the mutants in kinases encoded in the same clusters. This suggests that CheYs work independently from their cognate kinases to promote biofilm formation (Fig. 8). A similar situation was reported in *Shewanella oneidensis*, where it was shown that phosphorylated CheY3 is essential for

biofilm formation and that its phosphorylation comes from a different kinase than its cognate kinase CheA3 (83). They also showed that CheY3 interacts with Mxd proteins and the diguanylate cyclase PdgB. Mxd proteins are c-di-GMP effectors that belong to the glycosyl transferase family and are involved in polysaccharide production (84). Other signaling crosstalk has been proposed for *Agrobacterium tumefaciens*, where its CheY2 response regulator would be additionally phosphorylated by a different kinase than its own cognate kinase to modulate flagellar motility (85). Phenotypes of  $\Delta cheY$  mutants in PsPto correlate with low levels of intracellular c-di-GMP, suggesting their role in the control of the level of this second messenger. Low levels of c-di-GMP levels correlate with decreased biofilm and high motility, as the expression of flagellar genes and biofilm-related genes is inversely regulated (86, 87). Another explanation for the decreased biofilm observed in the  $\Delta cheY2$  mutant is that, since the transfer of the phosphoric group to the primary target is abolished, the CheA2 kinase could activate other response regulators like WspR. This is supported by the interaction observed between CheA2 and WspR. It is important to note that this finding does not contradict the impaired motility of the  $\Delta cheY2$  mutant. It would be expected that a strain with low levels of c-di-GMP had an increased motility. However, the lack of CheY2 impedes the transduction of signaling to the flagellar motor at the level of the interaction with FliM, resulting in a non-chemotactic strain regardless of its c-di-GMP levels.

Phenotypes of the  $\Delta wspR$  mutant strain correlate with those of the  $\Delta cheA3$  mutant strain, which relate with high c-di-GMP levels, suggesting a tandem activity of CheA3 kinase-WspR response regulator. Extensive work has been done in *Pseudomonas*, showing the WspR diguanylate cyclase activity as a response to the stimulation of the WspA chemoreceptor, resulting in increased c-di-GMP levels and biofilm formation (4, 66, 88–91). Our work about the ACF system does not align with most of the literature available on the topic. Armbruster et al. (92) reported that a deletion of *wspR* in *P. aeruginosa* resulted in similar amounts of biofilm relative to WT at later stages (24 hours), suggesting that any of the many other c-di-GMP cyclases present in this bacterium may ultimately compensate for c-di-GMP production in the absence of WspR (92). In the PsPto genome, there are 56 genes encoding putative c-di-GMP cyclases (93). These activities could account for a compensation activity in the  $\Delta wspR$  mutant background. Consequently, they might also contribute to compensatory mechanisms in the  $\Delta cheA3$  mutant, where the signaling pathway is impaired. However, other regulatory mechanisms leading to the modulation of the levels of c-di-GMP could be taking place.

BACTH analysis revealed unexpected interactions of WspR with both CheA1 and CheA2, which could explain the enhanced biofilm-related phenotypes observed in the  $\Delta cheA1$  and  $\Delta cheA2$  mutants. As in the case of the signaling through CheA3, if these two kinases also participate in keeping the homeostasis of c-di-GMP through WspR, their mutation could induce a compensatory effect. However, we cannot discard interactions between CheAs and other biofilm regulators. Previous data on *Comamonas testosteroni* also reveal the interaction of the chemotaxis kinase with two different response regulators, the chemotaxis response regulator CheY and the biofilm response regulator FlmD (22). We were not able to detect the interaction between the kinase CheA1 and the response regulator CheY1 in our assays. This fact, together with the independency in the control of phenotypes observed in mutant strains in these two proteins, suggests that they do not collaborate in the same signaling pathway (Fig. 8).

Our results showed that both the kinases and the response regulators belonging to F6, F8, and ACF pathways are needed for full virulence in tomato plants. All chemosensory systems are relevant in the stage involving an active entry process, as syringe-infiltration assays restored *in planta* bacterial population of mutant strains. However, the ACF system seems to have an additional role involving symptom development, as leaves inoculated with chemosensory mutants displayed reduced necrotic areas when compared to the WT. All mutant strains except  $\Delta cheY1$  are affected in their swimming abilities. This explains the defect in virulence, as PsPto requires active flagellar motility to achieve a successful infection/trigger infection (94).  $\Delta cheY1$  did not show a defect in

motility but a phenotype in biofilm formation. Since PsPto is a weak epiphyte, we could hypothesize that the epiphytic fitness of this strain is further compromised, rendering a lower infection ability.

We proposed a model of signal transduction and crosstalk among chemosensory systems in PsPto (Fig. 8). CheA2 would signal through the response regulator CheY2 to modulate flagellar rotation through its interaction with FlhM. Furthermore, CheA1, CheA2, and CheA3 would also interact with WspR, regulating biofilm formation and swimming. CheA1, CheA3, and WspR functions could modulate motility through indirect intermediates, such as c-di-GMP levels (89, 90). Our results also indicate how CheY1 and CheY2 response regulators do not align with their cognate kinases to regulate biofilm formation, where they act as biofilm promoters. These data suggest that both kinases and response regulators of PsPto interact with additional partners to control key traits involved in virulence. Further analyses are needed to unveil the complex regulation network associated with chemosensory functions in this bacterium.

Overall, we show for the first time the visualization of the F6 and F8 chemosensory systems of PsPto. Most of the chemoreceptors are assigned to the F6 system, which supports the chance for this visualization. The visualization of the F8 chemosensory system suggests that the only CR protein known to associate with it must have quite a relevant role in PsPto. Moreover, our results unveil the crosstalk among the F6, F8, and ACF systems at the level of the interactions between the CheA1 and CheA2 kinases with the response regulator WspR, pointing to this response regulator as a central sink of the chemosensory functions in PsPto for the control of virulence and related traits. This work shows novel results that contribute to the knowledge of chemosensory systems and their role in functions alternative to chemotaxis.

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Martí Munar-Palmer, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review and editing, Visualization | Saray Santamaría-Hernando, Formal analysis, Investigation, Methodology, Writing – review and editing | Janine Liedtke, Formal analysis, Investigation, Methodology, Writing – review and editing, Software | Davi R. Ortega, Data curation, Formal analysis, Methodology, Software | Gema López-Torrejón, Conceptualization, Writing – review and editing | José Juan Rodríguez-Herva, Conceptualization, Funding acquisition, Writing – review and editing | Ariane Briegel, Conceptualization, Funding acquisition, Writing – review and editing | Emilia López-Solanilla, Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review and editing

## ADDITIONAL FILES

The following material is available [online](#).

### Supplemental Material

**Supplemental material (mBio00871-24-S0001.docx).** Extended materials and methods; supplemental tables and figures.

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## ORIGINAL ARTICLE



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# *Pseudomonas syringae* pv. *tomato* infection of tomato plants is mediated by GABA and L-Pro chemoperception

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## Abstract

Foliar bacterial pathogens have to penetrate the plant tissue and access the interior of the apoplast in order to initiate the pathogenic phase. The entry process is driven by chemotaxis towards plant-derived compounds in order to locate plant openings. However, information on plant signals recognized by bacterial chemoreceptors is scarce. Here, we show that the perception of GABA and L-Pro, two abundant components of the tomato apoplast, through the PsPto-PscC chemoreceptor drives the entry of *Pseudomonas syringae* pv. *tomato* into the tomato apoplast. The recognition of both compounds by PsPto-PscC caused chemoattraction to both amino acids and participated in the regulation of GABA catabolism. Mutation of the PsPto-PscC chemoreceptor caused a reduced chemotactic response towards these compounds which in turn impaired entry and reduced virulence in tomato plants. Interestingly, GABA and L-Pro levels significantly increase in tomato plants upon pathogen infection and are involved in the regulation of the plant defence response. This is an example illustrating how bacteria respond to plant signals produced during the interaction as cues to access the plant apoplast and to ensure efficient infection.

## KEYWORDS

chemoreceptors, entry, GABA, L-Pro, *Pseudomonas syringae*, virulence

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## 1 | INTRODUCTION

The phyllosphere is a dynamic habitat where environmental conditions and nutrient availability vary spatially and temporally (Carvalho & Castillo, 2018; Sivakumar et al., 2020; Vorholt, 2012). This imposes a selective environment for bacteria where the ability to cope with adverse environmental conditions and to localize nutrients is a competitive advantage. For many bacterial pathogens, the phyllosphere provides a source of nutrients that are released from the plant interior through leaching and guttation but also through wounds in damaged leaves or via stomata (Ryffel et al., 2016; Vacher et al., 2016). Furthermore, stomata are the main entry points into the plant apoplast for many phytopathogenic bacteria (Melotto et al., 2008; Xin et al., 2018). Once inside the apoplast, the pathogenic stage is initiated. Disease development is thus conditioned by the ability to survive and adapt to the phyllosphere as well as to enter the plant apoplast efficiently. In both processes, chemotaxis plays a key role allowing bacteria to sense and respond to plant and environmental signals (Matilla & Krell, 2018).

Chemotaxis enables bacteria to move in response to chemical gradients (Adler, 1966). In a canonical chemotaxis signalling cascade, perception of a signal by the ligand-binding domain (LBD) of a chemoreceptor produces a molecular stimulus that alters the autokinase activity of the histidine kinase CheA, which in turn modulates transphosphorylation activity to the response regulator CheY (Matilla & Krell, 2018). When phosphorylated, CheY binds to the flagellar motor, altering its activity. Chemotaxis thus allows bacteria to rapidly adapt to changes in the environment and to actively move towards nutrient sources or signal molecules that inform the bacteria on their environment like hormones or quorum-sensing molecules (Alexandre et al., 2004; Schulze-Lefert & Robatzek, 2006). In the context of plant infection, chemotaxis facilitates bacterial access to the apoplast through stomata or wounds and is critical to ensure efficient infection (Matilla & Krell, 2018). In phytopathogenic bacteria, the importance of chemotaxis for efficient plant infection is supported by abundant experimental evidence (Antúñez-Lamas, Cabrera-Ordóñez, et al., 2009; Antúñez-Lamas, Cabrera, et al., 2009; Cerna-Vargas et al., 2019; Hawes & Smith, 1989; Hida et al., 2015; Kumar Verma et al., 2018; Malamud et al., 2011; Matas et al., 2012; Santamaría-Hernando et al., 2020; Tumewu et al., 2020; Yao & Allen, 2006, 2007). The relevance of chemotaxis is also reflected by the fact that about 90% of phytopathogenic bacteria possess chemosensory genes (Ortega, Zhulin, et al., 2017), whereas such genes are only found in 47% of total bacteria (Sanchis-López et al., 2021). In addition, phytopathogenic bacteria harbour on average 27 chemoreceptor genes (Lacal et al., 2010), a number that is well above the bacterial average of 13 (Sanchis-López et al., 2021).

Chemotaxis has been observed in response to root exudates or xylem sap (Verma et al., 2018; Yao & Allen, 2006) and also to abundant compounds in the plant apoplast like amino acids, sugars, organic acids, or phenolic compounds released from wounds (Antúñez-Lamas, Cabrera, et al., 2009; Ashby et al., 1988; Brewster et al., 2016; Hida et al., 2015, 2017, 2019; Kamoun & Kado, 1990; McKellar et al., 2015;

Parke et al., 1987; Tumewu et al., 2020). However, there is scarce information about the signals that govern the switch from the epiphytic to the pathogenic stage in the phyllosphere.

*Pseudomonas syringae* pv. *tomato* DC3000 (PsPto) is a model bacterium to study the molecular mechanisms related to plant infection. This bacterium is the causal agent of bacterial speck in tomato plants and is considered a highly aggressive pathogen once inside the plant (Boureau et al., 2002; Xin & He, 2013). However, it is a weak epiphyte, suggesting that the perception and rapid response of PsPto to plant and environmental signals is critical to ensure entry into the plant apoplast. Stomata are the main entry points of PsPto into the plant apoplast, and previous studies have shown that PsPto is able to move towards open stomata (Melotto et al., 2006). It has also been observed that the mutation of *cheA2*, encoding CheA of the chemotaxis pathway, causes a loss of chemotaxis and reduced fitness on plant hosts (Cerna-Vargas et al., 2019; Clarke et al., 2016). This bacterium was found to perform chemotaxis towards amino acids, sugars, organic acids, and amides (Cerna-Vargas et al., 2019; Cuppels, 1988; Kim et al., 2007). Despite the importance of motility and chemotaxis for PsPto phyllosphere colonization and apoplast entry, only one of its 49 chemoreceptors has been characterized. This chemoreceptor, PsPto-PscA, binds and mediates chemoattraction to D-Asp, L-Asp, and L-Glu and is required for full virulence in tomato plants (Cerna-Vargas et al., 2019). These results highlight the relevance of amino acid perception in PsPto virulence. Moreover, the saturation of PsPto-PscA with D-Asp by its addition to the inoculum reduced virulence significantly. These data suggest that interference with key chemoreceptors is an alternative strategy to fight phytopathogens (Cerna-Vargas et al., 2019).

Amino acids are implicated in multiple plant processes, including plant defence (Yang et al., 2020), and are abundantly present in the apoplastic tissue, providing a rich environment for the development of phytopathogens (Rico & Preston, 2008). The amino acid composition in the apoplast varies depending on the physiological state of the plant and in response to abiotic and biotic stresses including bacterial infection (Farvardin et al., 2020; O'Leary et al., 2016). This suggests that phytopathogens use amino acids not only as nutrients but also as signals to mediate plant interaction.

After PsPto infection, the concentration of the nonproteinogenic amino acid gamma-amino-butyric acid (GABA) increases significantly in *Arabidopsis thaliana* apoplasts (Ward et al., 2010). It has been observed that elevated GABA concentrations down-regulate the expression of genes that encode the type III secretion system (T3SS), the main virulence factor in PsPto (Park et al., 2010). However, this regulation only occurs once the infection has been initiated (McCraw et al., 2016) and it has been speculated that it may coincide with the increased expression of genes involved in GABA catabolism, as observed in apoplastic populations of *P. syringae* 48 h after infection (Yu et al., 2013). These observations indicate that GABA not only acts as a nutrient for PsPto but also as a signal to regulate its virulence inside the apoplast.

Several chemoreceptors capable of perceiving GABA have been described in different *Pseudomonas* species. The *Pseudomonas*

*putida* McpG chemoreceptor specifically recognizes GABA (Reyes-Darias, García, et al., 2015), while PctC of *Pseudomonas aeruginosa* perceives preferentially GABA but also binds L-Pro and L-His with lower affinity (Rico-Jiménez et al., 2013). In the phytopathogen *P. syringae* pv. *tabaci* the McpG chemoreceptor is implicated in GABA perception (Tumewu et al., 2020), while in *P. syringae* pv. *actinidiae* (Psa) the PscC chemoreceptor binds GABA and L-Pro (Ehrhardt et al., 2021; McKellar et al., 2015). The LBD of the PsPto chemoreceptor PSPTO\_2448 shares 97.7% sequence identity with the LBD of the PscC chemoreceptor of Psa. Interestingly, in addition to GABA, accumulation of proline has also been observed in *Arabidopsis* plants after PsPto infection (Gupta et al., 2020). It has been reported that this amino acid also plays a key role in plant defence against abiotic and biotic stresses (Verslues & Sharma, 2010).

The PSPTO\_2448 chemoreceptor has a dCache\_1 LBD domain that contains an amino acid recognition motif conserved in bacteria, archaea, and eukaryotes (Gumerov et al., 2022), suggesting that the perception of amino acids is an important feature throughout the Tree of Life. Considering its conservation and the important role of amino acids as signals for both plants and PsPto, we investigated signal recognition by PSPTO\_2448 and its role during plant interaction.

## 2 | RESULTS

### 2.1 | PsPto-PscC binds GABA and L-Pro

To identify the ligands recognized by PsPto-PscC, its LBD was purified using affinity chromatography and submitted to thermal shift assays. This method records changes in the thermal stability of proteins

caused by ligand binding (Martin-Mora et al., 2018). Increases in the midpoint of the protein unfolding transition ( $T_m$ ) greater than 2°C are considered significant and indicative of binding. The  $T_m$  of the ligand-free recombinant protein was 35.1°C, and significant  $T_m$  increases were observed for both GABA and L-Pro (Figure 1a). In contrast, no significant  $T_m$  increases were observed for any of the compounds present in the PM3B phenotype compound array that contain amino acids and many other nitrogen sources (Figure S1). Isothermal titration calorimetry assays confirmed these results as the titration of PsPto-PscC LBD with GABA and L-Pro revealed binding with affinities of  $1.87 \pm 0.3 \mu\text{M}$  and  $4.52 \pm 0.4 \mu\text{M}$ , respectively (Figure 1b). Considering that the *P. aeruginosa* PctC chemoreceptor binds L-His in addition to GABA and L-Pro, we also performed microcalorimetric titrations with L-His. However, no binding heats were detected (data not shown). These results indicate that PsPto-PscC LBD binds specifically GABA and L-Pro, as observed for the Psa homologue PscC (McKellar et al., 2015).

### 2.2 | PsPto-PscC mediates chemotaxis to GABA and L-Pro in PsPto

To evaluate the chemotactic response of PsPto to GABA and L-Pro, we performed quantitative capillary chemotaxis assays with the wild-type (WT) strain. The results showed significant responses of PsPto towards these compounds with a maximum chemotactic response at 1 mM (Figure 2). To evaluate whether the chemotactic responses towards these amino acids were mediated by the PsPto-PscC chemoreceptor, we generated a PsPto-*pscC* mutant strain. We observed reduced chemotaxis of the mutant strain to both ligands

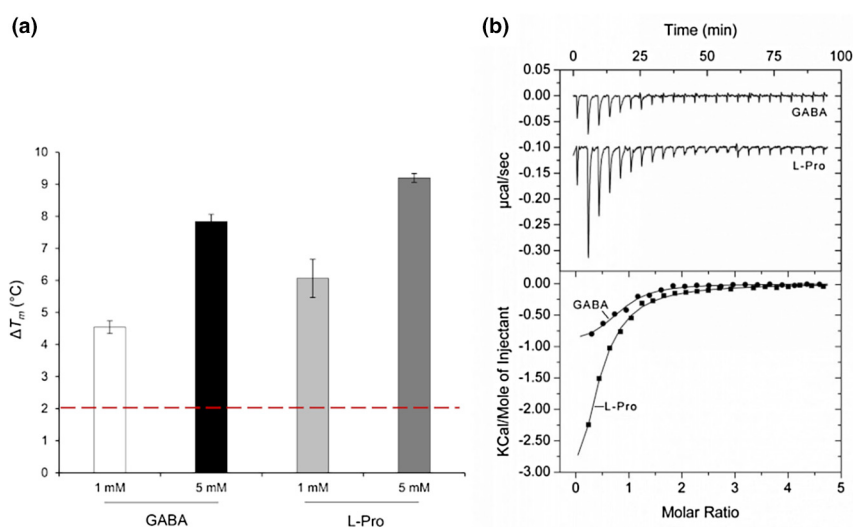


**FIGURE 1** The PsPto-PscC chemoreceptor binds specifically to GABA and L-Pro. (a) Differential scanning fluorimetry-based ligand screening of PsPto-PscC-LBD. Shown are the changes in the midpoint of the protein unfolding transition ( $T_m$ ) caused by the addition of 1 mM and 5 mM GABA or L-Pro with respect to the  $T_m$  of the ligand-free protein. The red dashed line highlights the threshold of 2°C, indicative of binding. Data are the means and standard deviations from three assays. (b) Microcalorimetric studies showing the binding of GABA and L-Pro to PsPto-PscC-LBD. Upper panel: Titration raw data for the injection of 4.2–8.6  $\mu\text{l}$  of 500  $\mu\text{M}$  ligand solutions into 13  $\mu\text{M}$  PsPto-PscC-LBD. Lower panel: Integrated, dilution heat-corrected, and concentration-normalized peak areas fitted with the one-binding-site model of ORIGIN.

at 1 mM, whereas at a ligand concentration of 5 mM a reduction was only observed for GABA (Figure 2). The observation that there was no complete loss of chemotaxis to both ligands in the PsPto-*pscC* mutant indicates the existence of other chemoreceptors with overlapping ligand specificities. To verify whether the ligand specificity determined by protein binding studies also corresponds to a chemotactic specificity, we conducted quantitative capillary chemotaxis assays using other amino acids such as L-Asp, L-Glu, and L-Asn. We did not find any significant differences in the chemotactic responses towards these compounds between the PsPto-*pscC* mutant and the WT strain (Figure S2). Complementation of the PsPto-*pscC* mutant with *pscC* provided in trans resulted in a chemotaxis phenotype that was superior in its magnitude to the WT strain (Figure S3). Expression in trans from multicopy plasmids enhances cellular chemoreceptor levels and previous studies following a similar approach have also reported an increase in the magnitude of the chemotactic response (Fernández et al., 2016; Hida et al., 2020; Rico-Jiménez et al., 2022).

### 2.3 | GABA perception by the PsPto-PscC chemoreceptor regulates the expression of genes involved in GABA catabolism

In *P. syringae* pv. *phaseolicola* NPS3121 and *P. syringae* pv. *syringae* B728a the expression of genes involved in GABA catabolism is up-regulated in the apoplast (Hernández-Morales et al., 2009; Yu et al., 2013). Considering that GABA is the most abundant amino acid in the tomato apoplast (Rico & Preston, 2008), we assessed whether the expression of genes involved in GABA catabolism was altered in PsPto following the addition of GABA to cells growing in *hrp*-inducing minimal medium. For these experiments we used GABA at 1 mM, a value close to its concentration in the tomato apoplast of 575  $\mu$ M (Rico & Preston, 2008). We observed that the expression of the gene encoding the permease GabP was up-regulated as compared to nontreated WT cells (Figure 3a). GabP has been identified as the principal GABA transporter in PsPto (McCraw et al., 2016).

In addition, we evaluated the expression of the three genes encoding GABA transaminases, *gabT1*, *gabT2*, and *gabT3*, and the succinic semialdehyde dehydrogenase gene *gabD*. Our results showed that the expression of *gabT1*, *gabT2*, and *gabD* was up-regulated after GABA addition compared to nontreated cells (Figure 3a). However, no differences in *gabT3* expression were observed (Figure 3a). These results show that the presence of GABA, in concentrations similar to those present in the tomato apoplast, alters the expression of GABA metabolic genes in PsPto. To determine whether this regulation is mediated by GABA perception through the PsPto-PscC chemoreceptor, we analysed the expression of these genes in the PsPto-*pscC* mutant strain. Whereas the gene expression of *gabT1*, *gabT2*, and *gabD* was down-regulated in the PsPto-*pscC* mutant strain as compared to the WT strain in the presence of GABA (Figure 3b), no differences in the expression of the *gabT3* and *gabP* genes were observed between the WT and the mutant strain (Figure 3b).

### 2.4 | Expression of *hrpL* and *avrPto* is not altered in the PsPto-*pscC* mutant

GABA levels increase in the apoplast under biotic stress conditions (Farvardin et al., 2020; O'Leary et al., 2016; Park et al., 2010). This increment has been associated with a greater plant resistance against PsPto infection (Deng et al., 2020; Park et al., 2010). Moreover, it has been reported that increased GABA levels are associated with a down-regulation in the expression of T3SS-related genes (McCraw et al., 2016; Park et al., 2010). Considering these previous results, we investigated whether the addition of 1 mM GABA causes a decrease in the expression of the *hrpL* and *avrPto* genes. In accordance with previous studies (McCraw et al., 2016), we observed a down-regulation in the expression of both genes as compared to nontreated WT cells (Figure 4a). In order to determine whether the regulation of the expression of the T3SS-related genes was mediated by GABA perception through the PsPto-PscC chemoreceptor, we analysed the expression of the genes in the mutant and WT

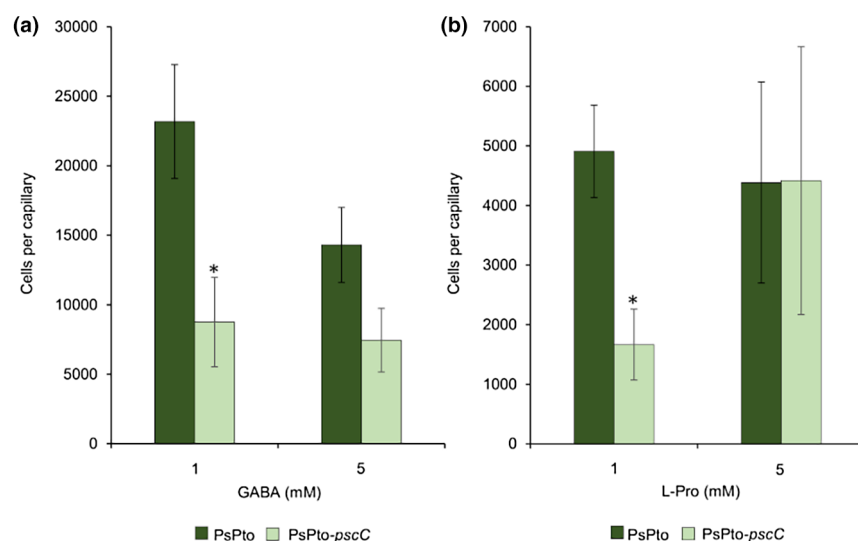


**FIGURE 2** Quantitative capillary chemotaxis assays of PsPto wild type and the PsPto-*pscC* mutant strains towards GABA (a) and L-Pro (b). The data have been corrected with the number of cells that swam into buffer-containing capillaries. Shown are means and standard errors from three independent experiments conducted in triplicate. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ).

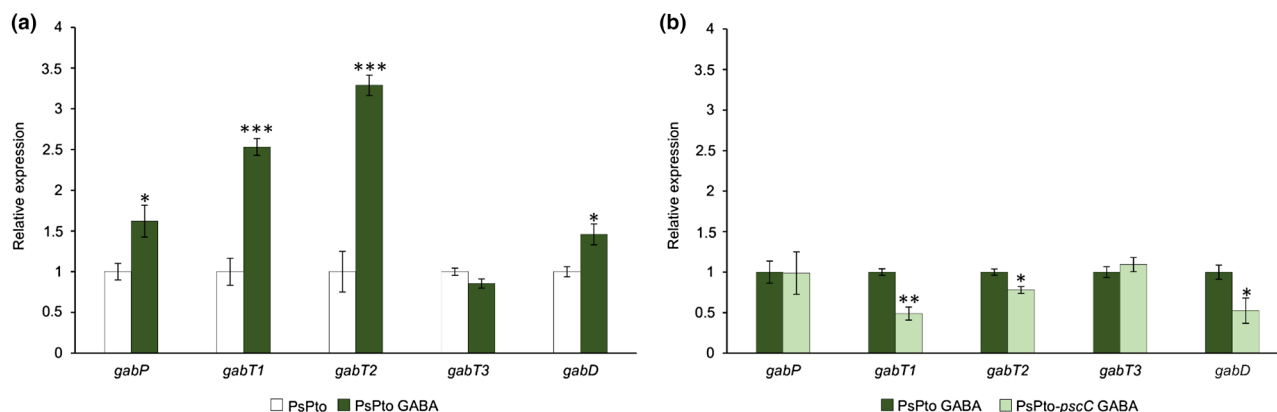


**FIGURE 3** Expression of GABA catabolic genes is regulated by GABA perception through the PsPto-PscC chemoreceptor. (a) Bars represent the relative expression changes, quantified by reverse transcription-quantitative PCR (RT-qPCR), in the wild-type strain *PsPto* in the presence (dark green) or absence (white) of 1 mM GABA. (b) Bars represent the relative expression changes, quantified by RT-qPCR, in the presence of 1 mM GABA in the *PsPto-pscC* mutant (light green) with respect to the wild-type strain (dark green). Data represent the means and standard errors of three biological replicates. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.005$ ).

strains but did not observe any significant difference (Figure 4b). The data thus indicate that PsPto-PscC mediates GABA chemoattraction, which in turn may facilitate access to the plant apoplast. However, PsPto-PscC does not seem to play a role in the regulation of T3SS-related genes that usually occurs during later stages of the infection.

## 2.5 | PsPto-PscC function is required for full virulence of PsPto in tomato plants

Chemotaxis allows bacteria to localize and move towards entry points such as stomata (Alexandre et al., 2004; Schulze-Lefert & Robatzek, 2006). Considering the reduced chemotaxis of the *PsPto-pscC* mutant strain to GABA and the observed alteration in gene expression, we evaluated whether these phenotypes had an effect on virulence in tomato plants. To this end, we conducted both entry and virulence assays to ascertain the role of this chemoreceptor during the initial or later stages of infection. Moreover, we used different inoculation methods, namely the spray-inoculation approach, which mimics natural entry conditions that bacteria face in the phyllosphere, and the infiltration method, in which the pathogen is placed inside the plant. There is a significant body of evidence demonstrating that chemotaxis-deficient strains show reduced virulence in spray-inoculation assays, which is due to that fact that directed motility is required to localize and move towards plant entry points (Yao & Allen, 2006). In a number of cases, analysis of the same chemotaxis mutant strains using infiltration assays, where the pathogen is directly introduced into the plant, did not reveal differences in virulence from the WT (Yao & Allen, 2006). Spray-inoculation resulted in a significant reduction in the number of *PsPto-pscC* mutant cells that entered the plant as compared to the WT cells at 2 h postinoculation (hpi) (Figure 5a). This finding is supported by a reduced cell count in virulence assays as measured 6 days postinoculation

(dpi) (Figure 5b). However, no significant differences between both strains were observed when plants were infiltrated (Figure 5b). The reduced entry and virulence of the *PsPto-pscC* mutant strain were restored to WT levels by complementation with the *pscC* gene provided in trans (Figures 5a and S4). These findings thus demonstrate that PsPto-PscC function is important to drive bacterial entry into the plant apoplast and that its mutation significantly alters infection severity in tomato plants.

In order to get further insight into the role of GABA chemoperception during the infection process, we assessed virulence in *A. thaliana gad1/2* mutant plants, which present reduced GABA levels (Scholz et al., 2015). Wild-type Col-0 and *gad1/2* *A. thaliana* plants were spray-inoculated with the WT and *PsPto-pscC* mutant strains. Data did not reveal any difference in bacterial populations between the WT and *PsPto-pscC* mutant strains, independently of the *Arabidopsis* genotype assayed (Figure 6). Moreover, as previously reported (Xian et al., 2020), no differences were observed in infection severity between Col-0 and *gad1/2* *A. thaliana* plants (Figure 6).

## 3 | DISCUSSION

Apoplastic fluid has traditionally been considered as an important source of nutrients for bacterial pathogens, allowing their growth and development. Moreover, bacteria have evolved adaptations to exploit changes in plant metabolism induced after microbe perception (Leonard et al., 2017), and, therefore, use apoplastic compounds as cues to regulate the interaction with the plant. Apoplastic compounds can also be considered as signals driving the access of bacterial pathogens to the plant apoplast (Antúnez-Lamas, Cabrera, et al., 2009; Melotto et al., 2006) and chemotaxis allows the motility-based entrance of bacteria (Matilla & Krell, 2018).

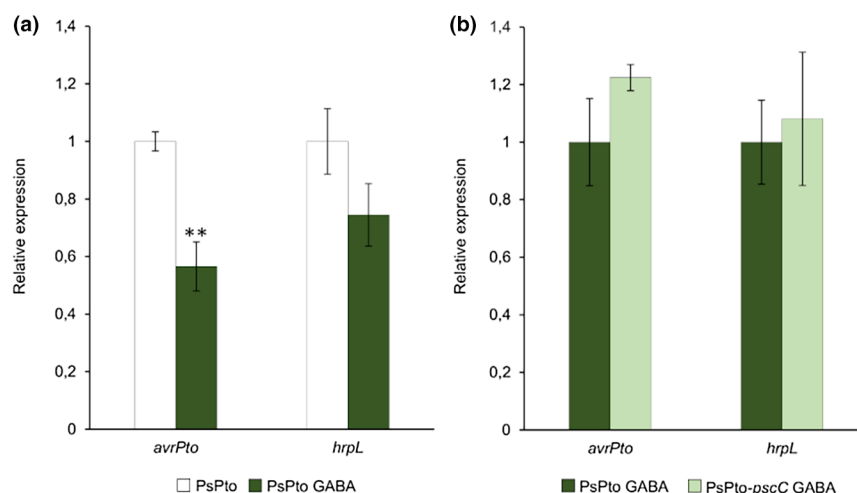


**FIGURE 4** The GABA-mediated down-regulation of the type III secretion system-related genes is independent of PsPto-PscC. (a) Bars represent the relative expression changes, quantified by reverse transcription-quantitative PCR, in the wild-type strain PsPto in the presence (dark green) or absence (white) of 1 mM GABA. (b) Bars represent the relative expression changes in the presence of 1 mM GABA in the PsPto-pscC mutant (light green) with respect to the wild-type strain (dark green). Data represent the means and standard errors of three biological replicates. Values that are significantly different are indicated by asterisks (\*\* $p < 0.01$ ).

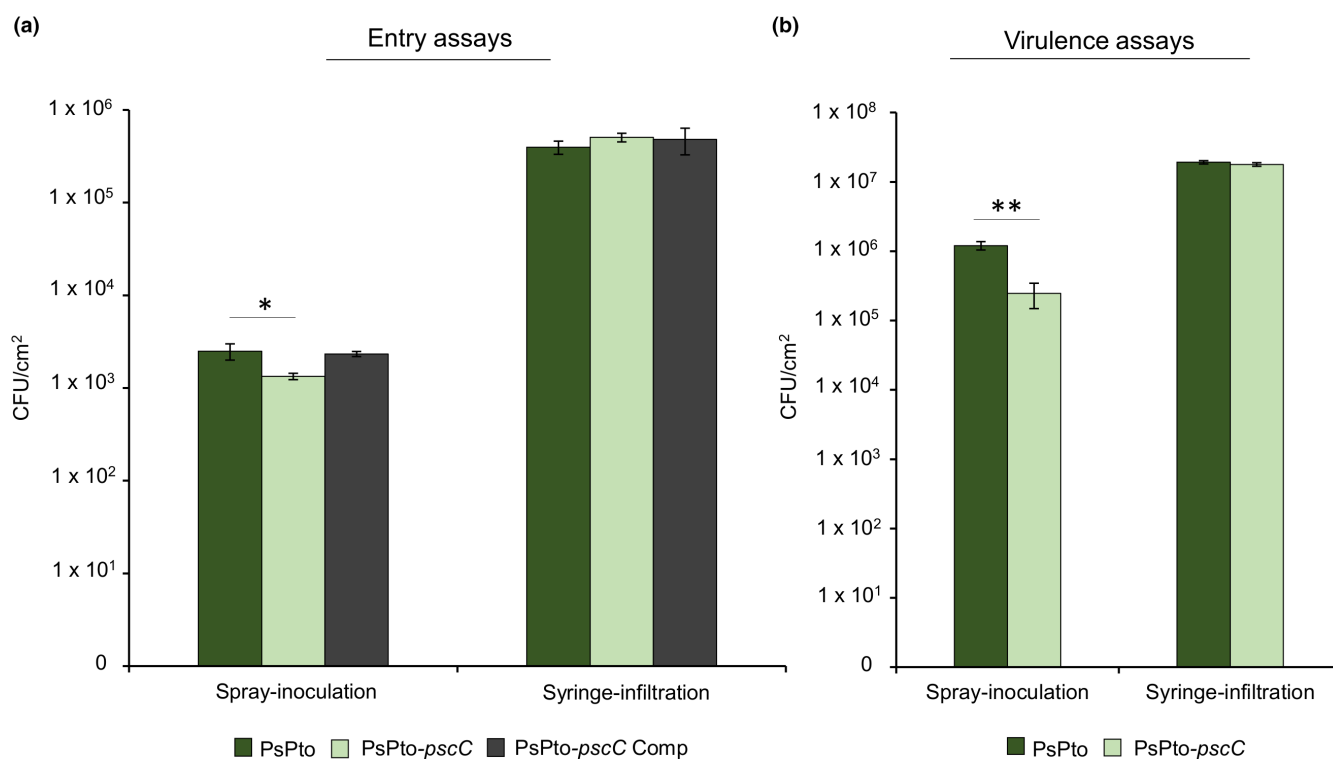


**FIGURE 5** The PsPto-pscC mutant shows reduced entry and virulence in tomato plants. (a) Entry of *Pseudomonas syringae* pv. *tomato* wild type (PsPto), PsPto-pscC mutant, and the complemented mutant (PsPto-pscC Comp) (10<sup>8</sup> cfu/ml) at 2 h postinoculation after spray-inoculation or syringe-infiltration. (b) Bacterial populations of the wild type and the PsPto-pscC mutant at 6 days postinoculation (dpi) after spray-inoculation (10<sup>8</sup> cfu/ml) or at 3 dpi after syringe-infiltration (3 x 10<sup>4</sup> cfu/ml). Shown are means and standard errors from three independent biological replicates. In each experiment, three plants were analysed by sampling five 1-cm-diameter leaf discs per plant. Values that are significantly different are indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ ).

Multiple lines of evidence indicate that the capacity to sense and respond to amino acids is of crucial importance for many forms of life. (1) A recent study has identified an amino acid-specific

sensor domain, dCache\_1AA, that is found throughout the Tree of Life in archaea, bacteria, and different eukaryotes (Gumerov et al., 2022). In bacteria, this domain is found in all major families

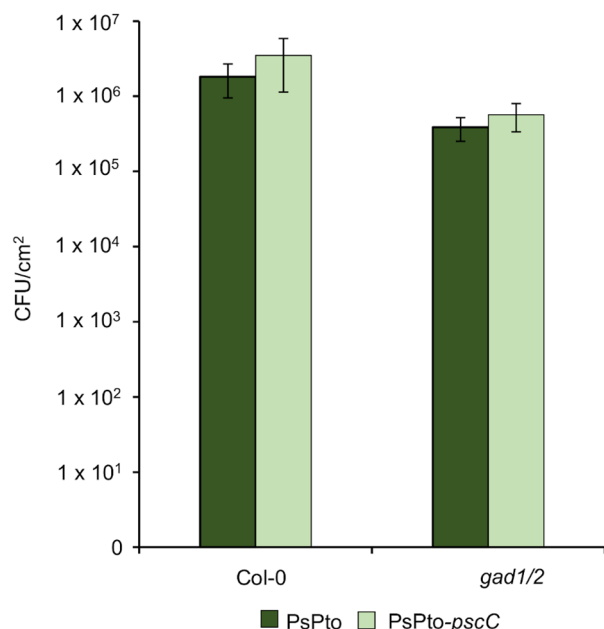


**FIGURE 6** Chemoperception of GABA is not required for *Arabidopsis* PsPto infection. Bacterial populations of wild-type (PsPto) and PsPto-pscC mutant strains at 6 days after spray-inoculation ( $3 \times 10^8$  cfu/ml) of *Arabidopsis thaliana* Col-0 or *gad1/2* plants. Shown are means and standard errors from three independent biological replicates. In each experiment, six inoculated plants were analysed by sampling three leaves per plant.

of transmembrane receptors, including chemoreceptors, sensor kinases, guanylate/adenylate cyclases, cyclic-di-GMP phosphodiesterases or serine/threonine kinases and phosphatases. (2) In bacteria, multiple mechanisms have evolved to sense amino acids. Next to a direct recognition at different types of LBDs, namely dCache\_1AA, TarH, or DAHL (Matilla, Velando, et al., 2021), there is evidence for transmembrane receptor stimulation by the binding of amino acid-loaded solute-binding proteins (Matilla, Ortega, & Krell, 2021). (3) The ligand profiles of many bacterial chemoreceptors have so far been determined (Matilla, Velando, et al., 2021). However, the by far most abundant family is formed by amino acid-responsive chemoreceptors that show a wide phylogenetic spread (Matilla, Velando, et al., 2021). In the framework of the general importance of amino acid sensing in life, our study illustrates the central role of sensing these ligands in the context of plant infection. In the context of phytopathogens, the amino acid composition of the apoplast and root exudates were found to change significantly after bacterial infection (O'Leary et al., 2016; Yuan et al., 2018). Taking this data together, amino acids may be key players in the signalling process between plants and bacteria during infection.

Only a limited number of amino acid-specific chemoreceptors have been functionally characterized in phytopathogenic bacteria (Cerna-Vargas et al., 2019; McKellar et al., 2015; Rico-Jiménez et al., 2013; Tumewu et al., 2020, 2021). In this work we have identified the ligands of the PsPto-PscC chemoreceptor of PsPto. We found that PsPto-PscC binds specifically to GABA and L-Pro, similar

to what was observed for the PscC homologue in *Psa* (McKellar et al., 2015). However, the characterization of the role of *Psa*-PscC during the interaction with the plant has not been addressed in *Psa*.

In plants, GABA and proline are implicated in the control of carbon/nitrogen metabolism and in the regulation of different aspects of plant physiology (Tarkowski et al., 2020). However, these amino acids also play a key role as signalling molecules during the plant response to different stresses, including pathogen infection (Seifikalhor et al., 2019; Tarkowski et al., 2020). Under stress conditions, GABA levels increase in the apoplast due to the activation of the glutamate decarboxylase enzyme GAD through  $\text{Ca}^{2+}$  signalling (Tarkowski et al., 2020; Ward et al., 2010). Similarly, L-Pro levels increase by the activation of the enzyme pyrroline-5-carboxylate synthase (P5CS), involved in L-Pro synthesis from L-Glu (Mattioli et al., 2009). Moreover, proline can be converted into GABA through a nonenzymatic reaction under stress conditions (Signorelli et al., 2015) and in the case of *Arabidopsis* plants, some GABA transporters are also able to transport proline (Breitkreuz et al., 1999). PsPto-PscC LBD bound GABA and L-Pro with high affinity as evidenced by  $K_D$  values in the lower micromolar range, which are comparable to those obtained for PscC from *Psa* (Ehrhardt et al., 2021) and PctC from *P. aeruginosa* (Rico-Jiménez et al., 2013). For PctA and PctB, two other amino acid-responsive dCache-containing chemoreceptors in *P. aeruginosa*, a correlation has been established between high ligand affinity and highly sensitive responses (Reyes-Darias, Yang, et al., 2015b). The capacity to mediate responses to low ligand concentrations may be a feature common to the GABA- and L-Pro-responsive chemoreceptors in pseudomonads.

The PsPto-PscC mutant showed some residual responses to both of its ligands, indicative of the existence of additional chemoreceptors that respond to these amino acids, a finding that may not surprise considering that PsPto encodes 49 chemoreceptors. Previous studies showed that GABA and L-Pro are not preferred nutrients for PsPto and that they are only used when the preferential nutrients have been consumed (McCraw et al., 2016). The described chemotaxis to these compounds, regardless of their nutritional value, may indicate that PsPto uses the perception of these amino acids as a cue to localize the apoplast and initiate infection. Similarly, the use of nonpreferred amino acids as signals to locate nutrients and start the interaction with the plant has been previously proposed for *Bacillus subtilis* (Yang et al., 2015).

The PsPto genome encodes three GABA transaminases and three succinate-semialdehyde dehydrogenases (Park et al., 2010). However, PsPto is unable to synthesize GABA directly from glutamate due to the lack of a GadB enzyme (McCraw et al., 2016). Therefore, it is tempting to speculate that this bacterium obtains GABA from the plant, particularly from the apoplast, where it is more abundant. The unusually high number of GABA catabolic enzymes in the PsPto genome may be related to a particular importance of GABA metabolism during the interaction with the plant host.

In a previous transcriptomic analysis carried out in *P. syringae* pv. *syringae* B728a, it was observed that the expression of *gabP*

and GABA catabolic genes was up-regulated in planta and that their expression was increased in cells growing in the apoplast as compared to epiphytic populations (Yu et al., 2013). We show here that the addition of GABA to PsPto growing in *hrp*-inducing minimal medium, increased *gabP* expression. GabP represents the main mechanism of GABA uptake in PsPto (McCraw et al., 2016). We also observed up-regulation of the encoding succinate-semialdehyde dehydrogenase (*gabD*) and transaminases (*gabT1* and *gabT2*), but not of *gabT3*. It has been suggested that the *gabT3* gene, found in a different location of the genome, may not be a GABA transaminase (Park et al., 2010). Interestingly, we observed that in the presence of GABA, the expression of *gabD*, *gabT1*, and *gabT2*, but not that of *gabP*, was down-regulated in the PsPto-*pscC* mutant strain, which suggests that the perception of GABA through this chemoreceptor regulates the expression of GABA catabolism genes.

The expression of T3SS genes, the main virulence factor in PsPto, is induced in the presence of plant-derived signals (Anderson et al., 2014; Tang et al., 2006). A recent work has reported that the two-component system AauS–AauR in PsPto directly connects the perception of host-derived amino acids (aspartate and glutamate) to the up-regulation of T3SS-encoding genes (Yan et al., 2020). Interestingly, the presence of increased levels of GABA down-regulates the expression of T3SS-related genes in PsPto (Park et al., 2010). Accordingly, we found that the expression of *avrPto* and *hrpL* genes was down-regulated in the WT strain in the presence of 1 mM GABA and that this regulation was not mediated by PsPto-*PscC*. In PsPto, the repression of T3SS-related genes is mediated by GABA uptake through the GabP permease (McCraw et al., 2016) and the expression of *gabP* was similar in the WT and the PsPto-*pscC* mutant strain. Interestingly, the expression of the *B. subtilis* GABA catabolic genes *gabT* and *gabD* is under the control of the regulatory protein GabR; however, the expression of *gabP* is not regulated by this protein (Belitsky & Sonenshein, 2002).

The genome analysis of PsPto indicates that it has four chemosensory pathways (Gumerov et al., 2019) and currently available data indicate that only one is involved in chemotaxis (Cerna-Vargas et al., 2019; Clarke et al., 2016). In *P. aeruginosa*, the four chemosensory pathways appear to be insulated signalling routes (Matilla, Martin-Mora, et al., 2021; Ortega, Fleetwood, et al., 2017). However, results have been reported that suggest a cross-talk between different chemosensory pathways in other species (Cerna-Vargas et al., 2019; Huang et al., 2019). Importantly, our previous analysis of the PsPto-*PscA* chemoreceptor strongly suggests the existence of interpathway communication (Cerna-Vargas et al., 2019). We demonstrated that deletion of the PsPto-*pscA* gene not only reduced chemotaxis to its cognate ligands, but also increased cyclic-di-GMP levels, which in turn modulated biofilm formation and swarming motility. We hypothesize that a similar cross-talk may account for the observation that the mutation of the PsPto-*PscC* chemoreceptor modulates the expression of genes involved in GABA catabolism.

We propose that PsPto-*PscC* has a role as GABA chemoreceptor in the early stages of plant interaction, namely the epiphytic phase, to locate entry sites and navigate towards them, ensuring apoplast invasion. Consistently, we show here that the reduced chemotactic response towards GABA and L-Pro observed in the mutant strain caused an impaired leaf entry and reduced virulence in tomato plants. We did not observe differences either in entry or in virulence between the WT and the mutant strain in *A. thaliana* plants. PsPto infected *A. thaliana* WT and *gad1/2* mutant plants, which produce lower GABA levels, in a similar manner. This is consistent with the proposition that PsPto virulence in *Arabidopsis* is not influenced by GABA (Xian et al., 2020), as it is the case in tomato plants. Under nonstress conditions GABA levels are lower in *Arabidopsis* than in tomato (Deng et al., 2020; Rico & Preston, 2008). Although GABA levels can suffer a threefold increase during biotic stress in *Arabidopsis* (Ward et al., 2010), the concentration is still well below that found in the tomato apoplast, which is around 500 µM (Rico & Preston, 2008) under non-stress conditions and may increase to 2.5 mM under biotic stress (Solomon & Oliver, 2001). These data suggest that the signals perceived by PsPto during infection of tomato and *Arabidopsis* are different.

Our data highlight the key role of GABA and L-Pro as plant-released chemoeffectors perceived by PsPto to drive entry into the tomato host plant. These results are in line with the suggested pivotal role of amino acid perception during bacterial plant infection. The differential role of this perception in *Arabidopsis* plants suggests that the specific profile of pathogen chemoperception functions is under evolutionary pressure of the presence of host-specific signals.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Bacterial strains, culture media, and growth conditions

Bacteria and plasmids used in this study are listed in Table S1. PsPto derivatives were grown at 28°C in King's B (KB) medium (King et al., 1954). *Escherichia coli* derivatives were grown at 37°C in lysogeny broth (LB) (Bertani, 1951). When appropriate, antibiotics were added to the medium at the following final concentrations: rifampicin 25 µg/ml, streptomycin 50 µg/ml, kanamycin 50 µg/ml.

### 4.2 | Construction of the expression plasmid for PsPto-*PscC*-LBD

The limits of the LBD domain of PsPto-*PscC* (PSPTO\_2448) were defined as the protein fragment between the two transmembrane regions as determined by the TMHMM server v. 2.0 (Krogh et al., 2001) and the DAS transmembrane region prediction algorithm (Cserzo et al., 1997).

A pET28b(+) derivative containing the DNA sequence of the PsPto-PscC-LBD, p2448-LBD, was purchased from Biocat. *E. coli* BL21(AI) (Invitrogen) was transformed with this plasmid for protein overexpression.

### 4.3 | Overexpression and purification of PsPto-PscC-LBD

*E. coli* BL21(AI) containing p2448-LBD was grown in LB medium supplemented with kanamycin at 28°C. Once the culture reached an OD<sub>600</sub> of 0.5, protein overexpression was induced by the addition of 0.05 mM isopropyl-β-D-1-thiogalactopyranoside and L-arabinose 0.2% (wt/vol). Growth was then continued overnight at 16°C prior to cell harvest by centrifugation at 6000 × g for 30 min at 4°C. Cell pellets were resuspended in buffer A (30 mM Tris.HCl, 300 mM NaCl, 10 mM imidazole, 10% [vol/vol] glycerol, pH 8.0), disrupted by sonication, and centrifuged at 13,000 × g for 1 h. The supernatant was then loaded onto a 5-ml HisTrap column (Amersham Biosciences) previously equilibrated with 5 column volumes of buffer A, which was subsequently washed with buffer A containing 10 mM imidazole and eluted with a linear gradient of 10 to 500 mM imidazole in buffer A. Protein-containing fractions were pooled and dialysed into buffer B (50 mM HEPES, 300 mM NaCl, 10% [vol/vol] glycerol, pH 8.0) for immediate analysis.

### 4.4 | Thermal shift assay-based high-throughput ligand screening

Thermal shift assays were performed on a Bio-Rad MyIQ2 real-time PCR instrument using 96-well plates. For ligand screening, the compound array PM3B (nitrogen sources) from Biolog (<https://www.biolog.com/wp-content/uploads/2020/04/00A-042-Rev-C-Pheno-type-MicroArrays-1-10-Plate-Maps.pdf>) was used. Assay mixtures (25 µl) contained 15 µM protein dialysed into buffer B, 5× SYPRO Orange (Life Technologies), and ligands at final concentrations of 1 and 5 mM (1 to 2 mM when using Biolog plates). In the ligand-free control well, the corresponding amount of buffer was added. Samples were heated from 23°C to 85°C at a scan rate of 1°C/min. The protein unfolding curves were obtained by monitoring the changes in SYPRO Orange fluorescence.  $T_m$  values were determined using the first derivatives of the raw fluorescence data.

### 4.5 | Isothermal titration calorimetry binding studies

Experiments were conducted on a VP-ITC microcalorimeter (MicroCal) at 25°C. PsPto-PscC-LBD was dialysed overnight against buffer B, adjusted to a concentration of 13 µM, and placed into the sample cell of the instrument. The protein was titrated by the injection of 4.2–8.6-µl aliquots of 500 µM ligand solutions that were

prepared in buffer B immediately before use. The mean enthalpies measured from the injection of ligands into buffer were subtracted from raw titration data prior to data analysis using the “one-binding-site” model of the MicroCal version of ORIGIN.

### 4.6 | Construction of PsPto-pscC mutant

To generate the PsPto-pscC mutant, an internal 502-bp fragment of *PSPTO\_2448* was amplified by PCR from the PsPto genome using the corresponding primers (Table S2). PCR products were cloned into pKOSac101 (Cerna-Vargas et al., 2019) and the resulting plasmid was introduced in *E. coli* CC118λpir, purified, and transferred into PsPto by electroporation. Plasmid integration was confirmed using PCR. For complementation assays, the chemoreceptor-encoding gene and its promoter region were amplified by PCR using the primers listed in Table S2 and the PCR product was cloned into the broad-host-range plasmid pBBR1MCS-2 (Kovach et al., 1995). The resulting plasmid was verified by DNA sequencing and transferred into the PsPto-pscC mutant by electroporation.

### 4.7 | Quantitative capillary chemotaxis assays

Bacterial cells were cultured overnight at 28°C in KB medium and diluted to an OD<sub>600</sub> of 0.05. At the early stationary phase of growth (OD<sub>600</sub> of 0.25), cultures were centrifuged at 1750 × g for 5 min, and the resulting pellet was washed twice with 10 mM HEPES, pH 7.0. Cells were then resuspended in the same buffer and adjusted to an OD<sub>600</sub> of 0.25. Subsequently, 230 µl of the cell suspension was placed into each well of a 96-well plate. One-microlitre capillaries were filled with the chemoeffector and immersed into the bacterial suspension for 30 min. Capillaries were removed from the bacterial suspension and rinsed with sterile water, and the content was expelled into 1 ml of NB medium (1 g/L yeast extract, 2 g/L beef extract, 5 g/L NaCl, and 5 g/L Bacto peptone). Serial dilutions were plated onto NB medium supplemented with the appropriate antibiotics, and the number of cfu was determined. In all cases, data were corrected by subtracting the number of cells that swam into buffer-containing capillaries.

### 4.8 | RNA extraction

Bacterial cells were grown at 28°C in *hrp*-inducing medium (Huynh et al., 1989) with shaking at 150 rpm to an OD<sub>600</sub> of 0.5. When necessary, GABA at a final concentration of 1 mM was added and cultures were incubated at 28°C for 2 h. Cells were then collected by centrifugation at 5000 × g for 5 min, the supernatant was discarded, and the pellets were stored at –80°C. Total RNA was extracted using TRI reagent solution (ThermoFisher Scientific) following the manufacturer's instructions. The purification was accomplished using the High Pure RNA Isolation Kit

(Roche) following the manufacturer's recommendations. RNA samples were quantified using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Inc.).

#### 4.9 | Reverse transcription-quantitative PCR

Total RNA was isolated as described above and converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers were designed to amplify fragments of approximately 100bp (Table S2) and the *rpoD* gene was used as an internal control (Sawada et al., 1999). Reverse transcription-quantitative PCR (RT-qPCR) amplifications were carried out on an ABI PRISM 7300 RT PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). Thermal cycling conditions were as follows: one cycle at 95°C for 10 min; 50 cycles at 95°C for 15 s and 60°C for 1 min; and a final cycle at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s. The relative gene expression ratio was calculated using the comparative critical threshold ( $\Delta\Delta C_t$ ) method (Pfaffl, 2001; Rotenberg et al., 2006).

#### 4.10 | Plant assays

*P. syringae* pv. *tomato* strains were grown at 28°C for 24 h on KB agar plates under dark conditions.

For virulence assays in tomato, cells were resuspended in 10 mM MgCl<sub>2</sub> and diluted to  $1 \times 10^8$  cfu/ml. Three-week-old tomato plants (*Solanum lycopersicum* 'Moneymaker') were spray-inoculated with the bacterial suspension. Silwet L-77 was added to the bacterial suspensions at a final concentration of 0.02% (vol/vol). Plants were infected at subjective dawn, which was at the end of the night cycle with immediate transfer to light. Plants were incubated in a growth chamber at 25°C at 60% relative humidity with a daily light period of 12 h. Six days after inoculation, the leaf symptoms were recorded, and bacterial populations were measured by sampling five 1-cm-diameter leaf discs per plant. Three independent biological replicates were performed. In each replicate three plants were used, and five independent leaves were sampled from each plant. The infected leaf discs were washed twice with 10 mM MgCl<sub>2</sub> prior to homogenization to eliminate the bacteria from the leaf surface. Plant material was homogenized in 10 mM MgCl<sub>2</sub> and drop-plated onto KB agar supplemented with the appropriate antibiotics. The average number of bacteria per cm<sup>2</sup>, isolated from five infected tomato leaves, was determined based on log-transformed data.

For the infiltration assays, leaves were syringe-infiltrated with a suspension containing  $3 \times 10^4$  cfu/ml, and three leaves were mock-infiltrated with 10 mM MgCl<sub>2</sub>. Bacterial populations were determined at 3 dpi following the same protocol described above for the spray-inoculation assay.

Tomato entry assays were performed as described above for the spray-inoculation and the syringe-infiltration assays, using a

bacterial suspension containing  $1 \times 10^8$  cfu/ml. Bacterial populations were determined at 2 h postinoculation. For entry assays, a surface sterilization procedure (Romero et al., 2019) was carried out prior to plant material homogenization. Briefly, leaves were surface-sterilized with a 5% (vol/vol) solution of commercial bleach and 0.01% (vol/vol) of Tween 20 for 10 min and finally rinsed three times with sterile distilled water. The final wash was plated on KB plates to confirm the absence of bacteria.

For virulence assays in *A. thaliana*, cells were resuspended in 10 mM MgCl<sub>2</sub> and diluted to  $3 \times 10^8$  cfu/ml. Four-week-old *A. thaliana* Col-0 or *gad1/2* double mutant (Scholz et al., 2015) plants were spray-inoculated with the bacterial suspension. The symptoms were recorded at 6 dpi. Bacterial populations on the leaves were determined by quantifying the average number of cfu isolated from three leaves. Plant material was processed as described for tomato plants.

#### 4.11 | Statistical analysis

Variables that were heteroscedastic and that did not follow a normal distribution were compared using generalized linear models. The remaining variables were analysed using general linear models. All analyses were performed using the statistical software package SPSS 25.0 (SPSS Inc.) and Centurion 18 (Statgraphics Technologies, Inc.).

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#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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