Review

Bacterial hybrid histidine kinases in plant–bacteria interactions

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Two-component signal transduction systems are essential for many bacteria to maintain homeostasis and adapt to environmental changes. Two-component signal transduction systems typically involve a membrane-bound histidine kinase that senses stimuli, autophosphorylates in the transmitter region and then transfers the phosphoryl group to the receiver domain of a cytoplasmic response regulator that mediates appropriate changes in bacterial physiology. Although usually found on distinct proteins, the transmitter and receiver modules are sometimes fused into a so-called hybrid histidine kinase (HyHK). Such structure results in multiple phosphate transfers that are believed to provide extra-fine-tuning mechanisms and more regulatory checkpoints than classical phosphotransfers. HyHK-based regulation may be crucial for finely tuning gene expression in a heterogeneous environment such as the rhizosphere, where intricate plant–bacteria interactions occur. In this review, we focus on roles fulfilled by bacterial HyHKs in plant-associated bacteria, providing recent findings on the mechanistic of their signalling properties. Recent insights into understanding additive regulatory properties fulfilled by the tethered receiver domain of HyHKs are also addressed.

Introduction

Bacterial cells have evolved sophisticated mechanisms to cope with transient changes in their environment. More particularly, signal transduction systems have been shown to regulate critical processes in bacteria. Two-component signal transduction systems (TCS) are one of the primary means by which bacteria sense and adapt to fluctuating environmental conditions. In its most basic form, the TCS signalling pathway relies on a phosphotransfer reaction between two proteins, generally a membrane-bound sensor histidine kinase (HK) and a cytoplasmic response regulator (RR) (Stock et al., 2000). Upon detection of a signal on the N-terminal variable region containing the input domain (e.g. PAS, GAF, CHASE and Cache sensory domains), HK monomers autophosphorylate on a conserved histidine residue located in the transmitter region and then transfer the phosphoryl group into a conserved aspartate residue located in the receiver (REC) domain of the RR (Fig. 1a). Once activated, the RR output domain elicits an appropriate response, typically by altering transcription of specific genes or allosteric regulation of target proteins (Galperin, 2010; Gao & Stock, 2010). Most two-component systems have a modular domain architecture organization allowing them to perform sophisticated and versatile regulatory strategies (Gao & Stock, 2009).

Phosphorelay systems represent more complex versions of canonical TCS. They generally consist of four signalling modules: (i) an HK transmitter domain, (ii) a REC domain, (iii) a histidine phosphotransfer domain (Hpt) and, finally, (iv) a second REC domain. The phosphotransfer reaction occurs in sequential steps (His from transmitter domain/Asp from REC domain/His from the Hpt domain/Asp from REC domain) (Fig. 1b) (Appleby et al., 1996). The first three modules are often combined in a single protein called hybrid HK (HyHK), thus containing one (and sometimes several) REC domains fused to its C-terminus (Fig. 1c). Consequently, the phosphoryl group is intramolecularly transferred to the intrinsic REC domain, shuttled to an Hpt domain and finally to a terminal RR. The Hpt domain has no catalytic activity and can be found either within the HyHK protein or as a stand-alone protein (Fig. 1c).

TCS signalling pathways are involved in the regulation of many important bacterial behaviours including metabolism, virulence, sporulation, quorum sensing (QS), antibiotic resistance, biofilm formation and stress response.

Abbreviations: c-di-GMP, bis(3′,5′)-cyclic diguanosine monophosphate; DSF, diffusible signalling factor; HK, histidine kinase; HyHK, hybrid histidine kinase; PCDWE, plant cell wall-degrading enzyme; PGPR, plant growth-promoting rhizobacteria; QS, quorum sensing; RR, response regulator; TCS, two-component signal transduction systems; Xcc, Xanthomonas campestris pv. campestris.
TCS genes are found in nearly every sequenced bacterial genome, with some encoding more than 200 (Borland et al., 2015; Whitworth & Cock, 2008). The prevalence of these systems underscores their astounding versatility and their utility in bacteria, as this variation in TCS gene number appears to be strongly correlated with the prevalent environmental conditions, with bacteria having the largest number of TCS genes being those exhibiting sophisticated interactions (e.g. myxococcales and cyanobacteria) and those that are ubiquitous (e.g. Pseudomonas aeruginosa) (Galperin, 2005; Rodrigue et al., 2000; Ulrich et al., 2005). With increasing research on TCS signalling mechanisms over the past 30 years, it is now acknowledged that TCS are not just simple stimulus–response systems but that their regulation is far more complex, embedded in more sophisticated regulatory networks (Jung et al., 2012). Indeed, many TCS involve multiple components, interconnections or cross-talk with other regulatory circuits, additional partners such as auxiliary proteins that modulate kinase and/or RR activities (Buelow & Raivio, 2010; Laub & Goulian, 2007; Mitrophanov & Groisman, 2008).

It is generally thought that multi-step phosphorylases may offer more regulation sites, more potential junctions for signal integration as well as the generation of fine-tuned responses (Appleby et al., 1996). HyHKs are found in over 50 % of all sequenced bacterial genomes, and nearly 25 % of all bacterial HKs are hybrids (Wuichet et al., 2010). Intriguingly, the majority of TCS found in lower eukaryotes (yeasts, fungi, slime moulds and plants) involve HyHKs and phosphorylases (Defosse et al., 2015). Phosphorylases have been described in the control and fine-tuning of several developmental processes that need to be orchestrated in both time and space, such as sporulation in Bacillus subtilis, cell cycle progression in Caulobacter crescentus and multicellular differentiation in Myxococcus xanthus (Biondi et al., 2006; Burbulis et al., 1991; Wegener-Feldbrugge & Sogaard-Andersen, 2009).

Extra-regulatory properties enabled by phosphorylases or more generally HyHKs may represent a powerful way of coping with a wide array of environmental cues, and especially those encountered in heterogeneous and rapidly fluctuating environments. In that respect, bacteria evolving in the rhizosphere, i.e. the narrow volume of soil influenced by plant root activity, face a highly heterogeneous environment due to the concomitant activity of plant roots and microbes, and must adjust their physiology quickly to survive (Hinsinger et al., 2005). Despite their contrasting effects on plant health, a common series of events, coordinated by host and bacterial signal molecules, underlies the development of successful bacteria–plant interactions (Hentschel et al., 2000). First, rhizosphere bacteria must survive and compete in the root environment where extensive competition for nutrients and space occurs (Kuz yakov & Xu, 2013; Lopez-Guerrero et al., 2013). Then, rhizobacteria must display a chemotactic response towards root exudates to reach the root surface of their host plant, to which they usually adhere via a process referred to as root colonization, and overcome plant defence responses (Drogue et al., 2012). Finally, an effective interaction can take place leading to enhanced plant growth (symbiosis), plant protection against pathogens (biocontrol) or plant disease (pathogenesis).

Notably, bacterial phosphorylases and especially HyHK-based regulation (the focus of this review) may be crucial for regulating gene expression in the rhizosphere in the right place at the right time, and especially during the interplay occurring throughout bacteria–plant interactions. Here, we review recent insights into the mechanistic understanding of bacterial HyHK signalling properties and emphasize the role played by bacterial HyHKs in the rhizosphere, using selected systems in different plant-associated rhizobacteria. Where data are available, the review shows that, besides its role in promoting phosphorylase reactions, the tethered REC domain found in HyHKs can fulfill other regulatory functions missing in classical HKs.

Role of hybrid HKs in chemotaxis regulation pathways

Plant roots exude a wide diversity of compounds, some of these acting as signals triggering movement of rhizobacteria towards the roots and eliciting accurate bacterial gene expression responses in this environment (Drogue et al., 2013). Classical HK and some HyHKs are involved in this so-called chemotaxis response. Although chemotaxis is widely used by rhizobacteria, allowing them to move towards root exudates, and is considered as a pivotal mechanism for successful root colonization (Bais et al., 2006), the role of HyHKs in this process has been more specifically investigated in Azospirillum. Strains of this genus are well-known plant growth-promoting rhizobacteria (PGPR) that live in close association with numerous host plants and have the capacity to increase crop yield (Wisniewski-Dyé et al., 2013).

The chemotaxis pathway commonly involves the perception of environmental signals by chemoreceptors. This information is transduced to CheA HK, which responds by autophosphorylation on a highly conserved histidine residue, and by phosphorylation of the CheY and CheB proteins on conserved aspartate residues (Fig. 1d). Rotation of the flagellum is consequently changed into response upon binding of the phosphorylated CheY at the flagellar motor. CheR and phosphorylated CheB, respectively acting as methyltransferase and methyl esterase, are involved in covalent modification of chemoreceptors (i.e. methylation/de-methylation), which is required for signal perception. This classic chemotaxis pathway was initially characterized in E. coli and Salmonella typhimurium (Parkinson et al., 2015). However, chemotaxis pathways are sometimes more elaborate, involving HyHKs proteins such as in Azospirilla PGPR (Fig. 1d).
Azospirillum strains contain multiple chemotaxis systems that were classified as Che1, Che2, Che3 and Che4 in Azospirillum brasilense (Hauwaerts et al., 2002; Scharf et al., 2016; Wisniewski-Dyé et al., 2011). Among the che clusters, cheA genes encode either classical HK or HyHK proteins. For instance, a survey of sequenced genomes of Azospirillum lipofermum 4B and A. brasilense Sp245 evidenced five and four distinct cheA genes, respectively, but only two corresponding to HyHKs (i.e. AZOLI_p20364/AZOBR_p1100035 and AZOLL_p40444/AZOBR_p1130073) (Wisniewski-Dyé et al., 2011). In A. brasilense Sp7, a chemotaxis cluster containing a cheA gene encoding an HyHK, sharing 98% identity with AZOBR_p1130073, was involved in chemotaxis towards different sugars and amino acids (e.g. malate, succinate, galactose, fructose, alanine, glutamate and glycerol) (Hauwaerts et al., 2002). A. brasilense also has the capacity to monitor its intracellular energy levels through energy taxis, which allows bacteria to search for rhizosphere micro-habitats with low energy.

![Diagram of TCS modular domain architecture and phosphotransfer signalling mechanisms](http://mic.microbiologyresearch.org 1717)

**Fig. 1.** Schematic diagram representing TCS modular domain architecture and phosphotransfer signalling mechanisms. Sites of phosphorylation on histidine (H) and aspartate (D) residues and conserved amino acid residues (H, N, D, F and G) found in the transmitter domain (containing both HisKA and HATPase domains) are indicated. (a) Simple TCS that employ a classical HK and an RR. (b) Multi-step phosphorelay mediated by separated proteins, i.e. an HK (transmitter domain), an RR (REC domain), a protein containing a histidine phosphotransfer (Hpt) domain and a second RR (REC domain). (c) Multi-step phosphorelay that employs a hybrid HK containing an additional REC domain and an Hpt domain that can be found either within an HyHK or as a stand-alone protein (dashed line). (d) The *Escherichia coli* chemotaxis CheA HK is composed of five domains (P1 to P5). The P1 domain constitutes the Hpt domain, the P2 domain binds CheY and CheB and confers CheA signalling specificity, the P3 domain mediates homodimerization, the P4 domain is the kinase domain that phosphorylates the P1 domain upon ATP binding and the P5 domain regulates kinase activity in response to chemoreceptors. Two RRs compete for the CheA phosphoryl group, CheY, that controls flagellar motor switching and CheB that controls adaptation of chemoreceptors through methylation (for more details, see Wadhams & Armitage (2004)). Sometimes, CheA proteins harbour an additional REC domain (dashed line), thereby forming a CheA hybrid kinase (see the text for more information). Symbols are as follows: green arrows, input domain; purple rectangles, transmitter domain; blue rectangles, REC domain; grey arrows, output domain; orange squares, Hpt domain; pink rectangle, CheW domain.
hydron concentration, supporting optimal energy supply and suitable for nitrogen fixation (Alexandre et al., 2000). This happens through a still poorly understood signal transduction cascade occurring between electron transport systems and CheA proteins (Alexandre et al., 2004).

In A. brasilense Sp7, an HyHK called Org35 or CstA (sharing 99% identity with A. brasilense Sp245 AZOBBR_150097 but having no homologue in A. lipoferum 4B) has been characterized. CstA is composed of two N-terminal PAS domains, a transmitter domain with kinase activity and a C-terminal REC domain (Cui et al., 2011). Although CstA is not an element of the core chemotaxis machinery, its inactivation resulted in reduced chemotaxis ability compared to the WT, suggesting its involvement in regulating chemotaxis (Cui et al., 2011). CstA might also be involved in the regulation of nitrogen fixation; the PAS domain of CstA was indeed shown to interact with the N-terminal GAF domain of NifA, a master regulator of nitrogen fixation (see below), and might modulate NifA activity in response to the perception of a still unknown signal (Tu et al., 2006).

Intriguingly, chemotaxis pathways involving HyHKs are more often retrieved in organisms that adopt particular lifestyles, including cyst development such as in Azospirillum or in its closest aquatic free-living relative, Rhodospirillum centenum, where these proteins permit additional points of regulation (He & Bauer, 2014). Such an involvement of chemotaxis HyHKs in the development of cysts has recently been suggested for CstA of A. brasilense (Li et al., 2011) and reported in R. centenum (He & Bauer, 2014). Cyst cells are non-motile dormant cells, formed when cells suffer from nutrient limitation or other stresses, and they accumulate poly-hydroxybutyric acid granules in their cytoplasm facilitating their resistance to stressful conditions. A Che3 signaling cascade was shown to be involved in the control of this cell differentiation process in R. centenum, whereas the Che1 and Che2 cascades control, respectively, chemotaxis and flagella biosynthesis (He & Bauer, 2014). The Che3 cascade is composed of eight distinct components: an MCP3 methyl-accepting chemoreceptor; CheB3 and CheR3 methylating/demethylating proteins; two CheW3 linker proteins; CheA3 and CheS3 HyHKs; and a single-REC domain RR CheY3 protein. CheS3 and CheY3 constitute TCS that negatively control cyst development, CheS3 phosphorylating the CheY3 regulator (He et al., 2013). Under cyst-inducing growth conditions (starvation or dessication), MCP3 receives a signal that activates CheA3, which in turn phosphorylates the REC domain of CheS3; this leads to inhibition of the CheS3 kinase activity, CheY3 remains unphosphorylated and cyst formation is therefore repressed (He et al., 2013). Moreover, in addition to phosphorylation by CheA3, the phosphorylation status of CheS3 is regulated by the molar ratio of ATP/ADP+ATP. A 35% decrease in cellular energy reduces the phosphorylation level of the transmitter domain of CheS3, giving rise to cyst formation (He et al., 2015). Whether similar regulation systems controlling cyst development exist in Azospirillum is currently unknown.

**Hybrid HKs and oxygen sensing in rhizobia–legumes symbiosis**

Rhizobia are phylogenetically diverse soil α- and β-proteobacteria able to form nitrogen-fixing symbiosis with legumes. The rhizobia–legume interaction relies on an exchange of signal molecules between the partners that leads to the formation of root nodules where bacteria are converted into nitrogen-fixing bacteroids. Rhizobia facilitate plant nutrition through the acquisition of nitrogen sources in return for photosynthetically fixed carbon (Masson-Boivin et al., 2009). The rhizobia–legume interplay requires concerted gene expression in both partners. Many signalling cascades act within the nodule to ensure proper bacteroid differentiation and symbiotic nitrogen fixation. Oxygen is a major physiological signal involved in the coordinated expression of genes necessary for nodule development and symbiotic nitrogen fixation (Dixon & Kahn, 2004). Oxygen limitation within legume nodules is crucial to protect nitrogenase activity (whose subunits are encoded by nifHKD). In bacteroids, protection against the detrimental effects of oxygen is mediated by the synthesis of leghaemoglobin, a plant oxygen-carrying protein, and bacteroid respiration is facilitated by FixNOPQ, a cbb3-type oxidase with a high oxygen affinity (Preisig et al., 1993).

The paradigm for an oxygen-dependent circuit was first described in Sinorhizobium meliloti 1021 (Fig. 2a). At the top of the cascade is the membrane sensor HK FixL and its cognate DNA-binding RR FixJ. The sensor domain of FixL contains an oxygen-sensing haem PAS domain that results in a conformational change when bound to oxygen. Then, only in the absence of oxygen, FixL autophosphorylates and transfers its phosphoryl group to FixJ. This phosphorylation induces dimerization and relieves the inhibitory interface between the REC domain and the transcriptional activator domain of FixJ (Da Re et al., 1999). FixJ directly activates the expression of two intermediate transcriptional regulators, NifA (which regulates nifHKD) and FixK (which encodes a CRP/FNR homologue regulating the fixNOPQ operon) (Da Re et al., 1999; Foussard et al., 1997). FixJ also indirectly regulates the expression of the phosphatase FixT; FixT can prevent FixL phosphorylation to FixJ or accumulation of autophosphorylated FixL, and thus it impedes fixK and nifA expression under micro-aerobic conditions (Garnerone et al., 1999).

Homologues of fixJ, fixK and nifA regulatory genes have been identified in many rhizobial strains; however, the way in which rhizobia perceive and relay the oxygen status to downstream targets varies considerably among species and even among strains (Bobik et al., 2006; Dixon & Kahn, 2004; Terpolilli et al., 2012). FixL exhibits substantial distinct domain architecture among Rhizobiales (Fig. 3). First, many FixLs appear to be soluble HK rather than...
membrane-spanning, as in Bradyrhizobium and Mesorhizobium strains. Second, the PAS domain of FixL may lack the oxygen-sensing haem domain, such as FixL of Rhizobium etli CNPAF512. Third, an additive sensor domain may be present on the N-terminal side. Fourth, the most striking difference among FixLs is the presence of an additional intrinsic REC domain in some strains of R. leguminosarum, R. etli and Sinorhizobium fredii (Fig. 3h–k); in all these strains, fixL encodes a soluble orphan HyHK and no gene encoding a fixJ homologue has been identified in the vicinity of fixL or elsewhere in the genome.

In R. leguminosarum bv. viciae VF39, under low oxygen concentration, the HyHK FixL functions in a phosphorelay mediated by the internal REC domain, allowing the regulation of fixK and fnrN and encoding a second CRP/FNR homologue (Fig. 2b) (Boesten & Priefer, 2004); however, nifA regulation is independent of FixL and FixK. In R. etli CFN42, where several FixK-like regulators act to regulate two copies of the fixNOPQ operon, the internal REC domain of FixL was found to stimulate FixL autophosphorylation and to assist O2-dependent regulation (Sousa et al., 2013). Compared to other FixL proteins among Rhizobiales, the FixL of R. etli showed a low affinity for O2 (Kd of 738 µM in R. etli and 142 µM in B. japonicum). Since FixL can respond sharply to minor changes in O2 concentration at high oxygen levels, it may serve as an early alert for a decrease in oxygen concentration (Sousa et al., 2013).

The advantage for some rhizobia of using an intramolecular phosphorelay (HyHK FixL) rather than the FixLJ
TCS is difficult to apprehend since many other components of the regulatory cascade differ among strains. This diversity of regulatory networks may underlie a relatively fast evolution allowing adaptation to various physiological constraints (Dixon & Kahn, 2004).

Hybrid HKs in biocontrol traits of pseudomonads

PGPR are usually separated into two groups: phytostimulators, enhancing plant growth directly by providing nutrients and/or phytohormones (such as strains of the above-cited genus *Azospirillum*), and biological control rhizobacteria that protect plants from pathogens through either the production of antimicrobial compounds or the induction of plant defence reactions [for reviews, see Couillerot et al. (2009) and Haas & Défago (2005)]. To our knowledge, besides the role of HyHKs in chemotaxis and thereby in rhizosphere competence, involvement of HyHKs in regulation of phytostimulation has not received much attention to date. Rather, HyHKs were shown to be involved in regulating key biocontrol processes in fluorescent pseudomonads.
An intricate regulatory network among three hybrid HKs, GacS, LadS and RetS, controls biocontrol properties in beneficial pseudomonads

Many fluorescent pseudomonads are biocontrol PGPR that protect plant roots from diseases caused by fungi, oomycetes and nematodes. Protection against plant diseases is mediated by the production of a range of antimicrobial secondary metabolites and lytic enzymes and by the induction of systemic resistance in the plant (Couillerot et al., 2009). Secondary metabolites include the antimicrobial compounds 2,4-diacetylphloroglucinol, hydrogen cyanide, pyoluteorin, phenazine, pyrrolnitrin, lytic exoenzymes, siderophores and cyclic lipopeptides (Gross & Loper, 2009; Haas & Keel, 2003).

The Gac/Rsm (global activator of antibiotic and cyanide synthesis) regulatory network is the key regulator of secondary metabolite production. It is well conserved among Pseudomonas spp. and in many Gammaproteobacteria, regulating a variety of essential processes that contribute to virulence in both animal- and plant-pathogenic pseudomonads, as well as to plant protection in beneficial root-associated fluorescent pseudomonads (Heeb & Haas, 2001; Lapouge et al., 2008; Sall et al., 2014). In biocontrol pseudomonads, the Gac/Rsm system has also been implicated in the regulation of many fundamental physiological processes, such as oxidative stress response (Heeb et al., 2005), carbon metabolism (Lapouge et al., 2008), swimming and swarming motilities (Kay et al., 2005; Martinez-Granero et al., 2012) and iron homeostasis (Hassan et al., 2010).

Central to the Gac/Rsm signalling cascade is the Gac TCS, which comprises the membrane-embedded HyHK GacS and its cognate RR GacA (Fig. 4). In response to a yet unidentified signal, GacS autophosphorylates and activates its cognate RR GacA through a phosphorelay mechanism (Goodman et al., 2009; Zuber et al., 2003). In turn, phosphorylated GacA activates the transcription of several regulatory small RNAs, termed rsmX, rsmY and rsmZ (Kay et al., 2005). These small RNAs bind and sequester the two RNA-binding proteins RsmA and RsmE (and in some strains, RsmI), resulting in the dissociation from their target mRNAs and suppression of translational repression (Kay et al., 2005; Reimmann et al., 2005). In Pseudomonas protegens CHA0, mutants simultaneously lacking rsmX, rsmY and rsmZ showed reduced biocontrol activity for protection of cucumber from the oomycete Pythium ultimum, a phenotype akin to that of gacS and gacA mutants (Kay et al., 2005).

Further complexity is added to these TCS by two unusual HyHKs that converge onto the Gac/Rsm system. These two HyHKs, termed RetS (regulator of exopolysaccharide and Type III Secretion) and LadS (loss of adhesion sensor) are orphan sensor kinases (i.e. not genetically associated with a cognate RR) that antagonistically impact the activity of the GacS sensor through a mechanism that is independent of phosphotransfer activity (Goodman et al., 2009; Ventre et al., 2006; Workentine et al., 2009). In the opportunistic human pathogen P. aeruginosa, RetS and LadS participate in the genetic switch controlling the transition from acute to chronic infection (Goodman et al., 2009; Ventre et al., 2006). RetS binds GacS through a direct and specific protein interaction involving their two transmitter domains, thus forming inactive RetS/GacS heterodimers (Goodman et al., 2009); this heterodimerization blocks GacS autophosphorylation and prevents GacA activation and downstream signalling, resulting in an increase of Type III Secretion system activity and repression of biofilm formation (Goodman et al., 2009; Vincent et al., 2010). In Pseudomonas fluorescens, both RetS and LadS were shown interact directly with GacS in a bacterial two-hybrid assay (Workentine et al., 2009). However, it still remains unclear how LadS positively impacts GacS activity. More recently, another membrane-bound orphan HyHK, PA1611, has been demonstrated to feed onto the Gac/Lad/RetS pathway in P. aeruginosa; PA1611 directly interacts with RetS in vivo via its transmitter domain, counteracting the RetS repressive role on GacS (Kong et al., 2013). Interestingly, homologues of PA1611 sharing identity levels higher than 65 % and with conserved domain architecture are encoded in genomes of fluorescent pseudomonads, suggesting that the involvement of PA1611 as an additional HyHK modulating the outcome of the GacS/RetS/LadS network might be widespread among members of this genus.

The environmental cues that trigger the activation of this multi-sensor regulatory network of HyHKs are unclear. In P. protegens, the GacS/GacA system is activated when cells reach high density, particularly during the transition from exponential to stationary cell phase (Kay et al., 2005). Indeed, expression of rsmZ is enhanced by the addition of extracts recovered from P. protegens CHA0 late exponential culture (Heeb et al., 2002). Besides, addition of these culture supernatants to mutants lacking gacA or rsmZ resulted in reduced expression of target genes, suggesting that the Gac/Rsm system is also involved in regulation of the production of these signals (Kay et al., 2005). However, the nature of the inducible signals perceived is still under investigation, although they are not related to the well-known QS signals such as N-acyl homoserine lactones or type 2 auto-sinducer (Heeb et al., 2002). By this mechanism, biocontrol factors are produced only when cell density becomes high enough to ensure successful host plant colonization, and the positive feedback loop enables rapid switching of cells primary to secondary metabolism.

LadS and RetS are modular sensors that belong to the family of 7TM receptors characterized by a signalling domain consisting of an N-terminal 7-transmembrane region and a periplasmic sensor domain (diverse intracellular signalling module extracellular 2) (Fig. 4) (Goodman et al., 2004; Ventre et al., 2006). Crystal resolution structure of P. aeruginosa RetS periplasmic domain diverse intracellular signalling module extracellular 2 (RetS) revealed two distinct ligand-binding sites, with one showing a β-sandwich fold reminiscent of carbohydrate-binding modules found in other proteins including LadS (Vincent et al., 2010). RetS and LadSp share only moderate sequence identity (35 %),
suggesting that they may bind similar but distinct carbohydrate-like ligands commonly found in plant root exudates (Vincent et al., 2010). RetS seems to act as a temperature-sensitive sensor in fluorescent pseudomonads. In P. protegens CHA0, elevated temperature (around 35°C) has a negative impact on production of 2,4-diacetylphloroglucinol and hydrogen cyanide by comparison with their production at 30°C (Humair et al., 2009). This temperature sensitivity is dependent on RetS, as expression of rsmX, rsmY and rsmZ genes was not affected by temperature in either a gacS or a ladS mutant background, whereas in a retS mutant, all three genes were expressed at a high level and temperature sensitivity was lost (Humair et al., 2009). Taken together, these observations led to a model in which elevated temperature resulted in a change in membrane fluidity, leading to a stronger

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**Fig. 4.** Role of the GacS/LadS/RetS HyHK sensor network in the regulation of biocontrol properties in fluorescent Pseudomonas. Under the influence of various stimuli (indicated by lightning bolts), LadS and RetS directly interact with GacS, thereby modulating GacS kinase activity and ultimately GacA transcriptional control of the expression of the three small RNAs, RsmX, RsmY and RsmZ. These three small RNAs bind and sequester the two RNA-binding proteins, RsmA and RsmE, which counter their translational repression on target mRNAs encoding proteins involved in root colonization and biocontrol efficiency. See the text for further explanation. Symbols are as follows: purple squares, transmitter domain; blue squares, REC domain; orange squares, Hpt domain; grey arrow, output domain; ?, unknown. Black arrows and bar-headlines respectively are positive and negative effects (not necessarily direct). Dotted arrows and headlines indicate a lack of functional validation. Abbreviations: PP, periplasm; CM, cytoplasmic membrane; CP, cytoplasm.
RetS–GacS interaction and therefore in inhibition of exoprotein production (Fig. 4). Thus, considering that the growth of many competitive micro-organisms is inhibited when the soil temperature reaches 35°C, as well as the high energetic burden necessary to produce secondary metabolites, temperature responsiveness may allow fluorescent pseudomonas to fine-tune the switch from primary to secondary metabolism.

In P. protegens CHA0, expression of secondary metabolites is also controlled by Krebs cycle function; mutation in pycAB (encoding pyruvate carboxylase) and in fumA (encoding fumarase isoenzyme) led respectively to down-regulation and up-regulation of expression of the GacA-dependent small RNAs and production of secondary metabolites (Takeuchi et al., 2009). Interestingly, these effects were shown to rely upon GacS but do not implicate RetS or LdS (Takeuchi et al., 2009). The alarmon guanosine tetraphosphate (ppGpp) that accumulates when cells sense nutrient starvation or stress [for a recent review, see Hauryliuk et al. (2015)] was also shown to be connected with the Gac/Rsm signal transduction system (Takeuchi et al., 2012). Indeed, ppGpp concentration was found to be, respectively, higher in a gacA mutant and lower in a retS-deficient strain relative to WT. Likewise, in a relA spoT double mutant in which ppGpp production was completely abolished, expression of rsmY and rsmZ was reduced, leading to decreased antibiotic production, root colonization and biocontrol efficacy in a cucumber–P. ultimum pathosystem (Takeuchi et al., 2012). In sum, when nutrients become scarce or under stress conditions, accumulation of ppGpp activates the Gac/Rsm cascade, allowing cells to excrete secondary metabolites that protect them from deleterious competing micro-organisms in the rhizosphere.

Altogether, convergence between these different HyHKs results in a high degree of intricacy in the regulatory networks that finely control the transition from primary to secondary metabolism in the rhizosphere. Undoubtedly, direct interaction between these HyHK sensors provides additional levels of control where both extracellular and intracellular inputs are integrated, without the need for cross-phosphorylation between sensors and non-cognate RR.

**FitF regulates insecticidal activity of root-associated pseudomonads**

Some biocontrol pseudomonads belonging to species P. protegens and Pseudomonas chlororaphis exhibit insecticidal activity against larvae of major agricultural insect pests (Kupferschmied et al., 2013; Péchý-Tarr et al., 2008, 2013; Ruffner et al., 2015). This insecticidal activity depends to a significant extent on the production of a large protein, the Fit toxin. The global regulator GacA is required for full insecticidal activity, but its deletion does not completely abolish insecticidal activity (Ruffner et al., 2013).

The Fit toxin gene (fitD) is part of a conserved locus composed of eight genes (fitABCDEFGH), with fitABC-E encoding a type I secretion system displaying striking similarities to RTX toxin transporters and predicted to contribute to toxin export and fitFGH encoding three regulatory proteins involved in regulation of toxinogenesis (fitF encoding an HyHK) (Ruffner et al., 2013). In the WT background of P. protegens CHA0, expression of the mCherry-tagged Fit toxin is activated at high levels in the insect host, i.e. when needed, but not on plant roots or in common laboratory media (Péchý-Tarr et al., 2013).

All three regulatory proteins were shown to be crucial for the observed tight regulation of toxin production in P. protegens CHA0. The LysR-type regulator FitG acts as a transcriptional activator and the RR FitH acts as a repressor of the expression of both the Fit toxin gene fitD and the type I transporter genes fitABC-E (Péchý-Tarr et al., 2013). Accordingly, a fitH mutant expressed the Fit toxin under all conditions. As for the HyHK FitF, inactivation of the corresponding gene abolished expression of fitD and decreased the virulence in a *Galleria* injection assay (Kupferschmied et al., 2014). The current proposed model implies that the HyHK FitF perceives a yet unknown signal and inactivates the RR FitH by phosphorylation upon infection of an insect (Fig. 5). As FitH acts as a repressor, its inactivation somehow releases FitG, leading to the activation of toxin expression (Kupferschmied et al., 2014). Whether the REC domain of FitF fulfills roles other than transferring phosphate has not yet been investigated.

The patchy distribution of the fit genes (found exclusively in genomes of some P. protegens and P. chlororaphis but not in closely related pseudomonads) and their phylogenetic incongruence indicate that the Fit cluster evolved via the acquisition of insecticidal elements by horizontal transfer followed by sequence rearrangements (Ruffner et al., 2015). Moreover, FitF shares a PAS-sensing domain with DctB, an HK regulating carbon uptake in Proteobacteria. FitF most likely acquired its specificity through domain shuffling followed by sequence rearrangements (Ruffner et al., 2015).

Hybrid HKs in plant–pathogen interactions

**VirA of Agrobacterium tumefaciens acts as an enhancing factor of the output response**

*A. tumefaciens* is the causative agent of crown gall tumours on dicotyledonous plants. One of the very first steps of pathogenesis depends on the VirA/VirG TCS encoded by the tumour-inducing plasmid (pTi). VirA, a membrane-bound HyHK, senses phenolic compounds (e.g. acetosyringone) released by wounded plant tissues in the presence of sugars and acidic pH and activates its
cognate RR VirG that acts through binding to the promoter of virulence genes [for review, see Brencic & Winans (2005)]. Once activated, virulence genes mediate the transfer of Ti-plasmid T-DNA from the pathogen to the host plant, resulting in tumour formation. Several lines of evidence, coupled to the fact that VirA possesses no Hpt domain, suggest that the intrinsic VirA REC domain does not participate in a phosphorelay (Mukhopadhyay et al., 2004) but might rather function as an enhancing factor for virulence gene expression (Wise et al., 2010) (Fig. 6). Indeed, deletion of the VirA REC domain does modify vir gene expression, but the observed effect varies significantly depending on how virG and virAΔR (resulting in a truncated version of VirA dispossessed from its REC domain) are expressed. Earlier studies using virAΔR and constitutively expressed virG showed vir gene expression in the absence of the normally essential phenolic inducer acetosyringone if a sugar was available, thus defining the VirA REC domain as an inhibitor of vir gene expression (Chang et al., 1996). More recent studies, with virG expressed solely from the Ti-plasmid, demonstrated that the VirA REC domain is essential for vir gene expression and accumulation of VirG (Wise et al., 2010). Using bacterial double-hybrid

**Fig. 5.** FitF regulation of insecticidal activity of root-associated pseudomonads. An insect signal stimulates the phosphotransfer cascade from the HyHK FitF to the cognate RR FitH that becomes inactive. As FitH acts as a repressor, its inactivation releases the LysR-type transcriptional activator FitG, thus allowing expression of the Fit toxin gene fitD and of the type I transporter genes fitABC-E. Green: PAS_3 input domain; purple: transmitter domain; blue, REC domain; grey, DNA-binding LysR output domain; light pink, substrate-binding LysR domain. Abbreviations: PP, periplasm; CM, cytoplasmic membrane; CP, cytoplasm; HTH, helix–turn–helix motif.
and pull-down assays, the VirA REC domain was shown to interact with the DNA-binding domain of VirG (Wise et al., 2010; Wise & Binns, 2016). Altogether, this suggests that the VirA REC domain is an enhancing factor of VirG expression and that, through a domain–domain interaction mechanism, this domain favours VirG recruitment and ultimately phosphotransfer from the VirA transmitter domain to the VirG receiver domain (Fig. 6). Moreover, the VirA REC domain appears also to have a role in limiting VirG activation to conditions that mimic those found at the site of a plant wound (i.e. presence of sugars and phenolics) (Wise & Binns, 2016). Indeed, in the presence of glucose but absence of acetosyringone, VirAΔAR was able to induce vir gene expression in a dosedependent manner whereas full-length VirA was inactive. This indicates that the tethered VirA REC domain controls phosphate transfer to VirG and suggests that the supplementary sugar signal increases autophosphorylation of VirA (Wise & Binns, 2016).

The Rcs phosphorelay controls biofilm formation and virulence in phytopathogenic enterobacteria in a temporal manner

The Rcs (regulator of capsule synthesis) phosphorelay was first described in E. coli for its implication in the regulation of the synthesis of capsular polysaccharide colonic acid (Stout & Gottesman, 1990). It is well conserved throughout the Enterobacteriaceae family, and further studies demonstrated that the Rcs system plays a much broader role in the transcriptional control of cell division, antibiotic resistance, motility, biofilm formation and pathogenesis in various enterics (Majdalani & Gottesman, 2005). In phytopathogenic enterobacteria, the Rcs phosphorelay governs many aspects of bacterial virulence. In the vascular wilt pathogens Erwinia amylovora and Pantoea stewartii, the Rcs system controls the production of capsular polysaccharides within xylem vessels, respectively amylovoran and stewartan, that obstruct water circulation and cause plant disease (Minogue et al., 2005; Wang et al., 2009). In the soft-rot bacteria
Dickeya dactylii and Pectobacterium carotovorum, the Rcs phosphorelay is involved in the regulation of plant cell wall-degrading enzymes (PCDWEs), such as pectinas, cellulases and proteases, that cause maceration of plant tissues (Andresen et al., 2007; Bouchart et al., 2010). In all cases, mutants affected in the Rcs system are highly impaired in their ability to infect host plants (Andresen et al., 2007; Bouchart et al., 2010; Carlier & von Bodman, 2006; Wang et al., 2009).

The Rcs phosphorelay is a signal transduction system that involves at least five proteins in E. coli: three ‘core’ signalling proteins, an inner membrane spanning Hpt (RcsC), a cytoplasmic DNA-binding RR (RcsB) and an intermediary phosphorelay protein (RcsD) and two accessory proteins (RcsF and RcsA). Upon receiving an appropriate signal, RcsF, a lipoprotein anchored in the outer membrane, activates RcsC via an as yet unknown mechanism (Castanié-Cornet et al., 2006). When activated, RcsC autophosphorylates and transfers its phosphoryl group onto its internal REC domain. From here, the phosphoryl group is transferred to the Hpt domain localized in the intermediate kinase RcsD and then to the conserved aspartate residue of the RR RcsB (Fig. 7). Finally, depending on its phosphorylation status, RcsB binds either alone to the target genes or together with the accessory protein RcsA to promoters of Rcs-regulated genes [for more details, see Clarke (2010) and Majdalani & Gottesman (2005)]. In both P. carotovorum and E. amylovora, RcsB positively activates the expression of virulence factors, respectively PCDWEs and amylovoran biosynthesis genes, and negatively regulates motility, through the binding of RcsB to the master operon of flagella synthesis, fliDC (Andresen et al., 2007, 2010; Wang et al., 2012).

Both RcsC and RcsD contain a structural domain termed ABL (Alpha/Beta/Loop) respectively located N-terminally to their receiver and Hpt domains (Fig. 7) (Schmöe et al., 2011). The RcsD ABL domain functions by increasing the affinity for RcsD to RcsB by interacting with the DNA-binding domain of the RR, thus resulting in the formation of complexes between RcsD-Hpt and RcsB-REC domains that favour phosphate flow (Schmöe et al., 2011). As a consequence, this mechanism may allow the fine-tuning of the concentration of phosphorylated RcsB. Furthermore, the RcsC Hpt domain was shown to have both kinase and phosphatase activities, directing the net flow of phosphate between RcsC–RcsD–RcsB and, as a consequence, modulating the strength of the output. These enzymatic activities have been shown to play a key role in the ability of E. amylovora to control amylovoran production in vitro and disease progression in planta (Wang et al., 2011). Indeed, expression of the amylovoran biosynthetic gene amnG was lower in an rcsC mutant background than in the parental strain during in planta infection of immature pear fruits, whereas expression of the asmG gene was higher in the rcsC mutant than in the WT strain in liquid culture (Wang et al., 2009, 2011). These observations imply that an in planta signal modulates the balance between RcsC kinase/phosphatase activities that finely adjust the level of phosphorylated RcsB and therefore ensure appropriate expression of virulence factors during plant infection.

A relatively large number of stimuli were found to activate the Rcs phosphorelay in various enterobacteria, with RcsC responding to a wide range of signals such as solid surface, osmotic stress, low temperature and dessication [for more details, see Clarke (2010) and Majdalani & Gottesman (2005)]. Auxiliary co-sensor proteins such as RcsF and IgaA (in Salmonella enterica) converge onto RscC (and/or RcsD) to either stimulate or inhibit its activity (Majdalani & Gottesman, 2005). Interestingly, all these conditions are related to perturbation of the cell surface envelope, and current consensus supports the hypothesis that perturbation of the integrity of cell membranes induces the Rcs regulon. Consistent with this assumption, transcriptome-based analyses of E. amylovora rcsBC regulon highlighted a high proportion of differentially expressed genes related to cell envelope biogenesis (Wang et al., 2012). Consequently, during host plant colonization, alteration in cell envelope integrity may increase the activity of the Rcs system, which in turn provides Erwinia (and more generally enteric phytopathogens) a way of avoiding plant defence recognition, through the remodelling of cell surface and enhanced production of virulence factors (Fig. 7). In addition, another major stimulus sensed by RscCDB is the increase of osmolarity in the medium. Indeed, in D. dactylii, osmolarity perception is achieved by an increase in the abundance of osmoregulated periplasmic glucans that are major periplasmic components of the envelope of most proteobacteria required for virulence, as the osmolarity of the medium decreases (Bouchart et al., 2010). Osmoregulated periplasmic glucans were found to control Rcs phosphorelay activity in a concentration-dependent manner (Bontemps-Gallo et al., 2013; Madec et al., 2014).

Several lines of evidence point towards a role for the Rcs phosphorelay in the temporal control of the lifestyle decisions governing biofilm formation and pathogenicity (Clarke, 2010; Huang et al., 2006). In E. coli, the Rcs system represses genes involved in surface attachment (i.e. flagella, curli, adhesin appendages) whereas it activates the expression of genes participating in biofilm maturation (i.e. capsular polysaccharides), thus suggesting that it controls the transition from early to late biofilm (Clarke, 2010; Huang et al., 2006). In E. amylovora, an rcsC mutant showed increased production of amylovoran but remained non-pathogenic on both apple and pear fruits (Wang et al., 2009). Furthermore, in D. dactylii, constitutive activation of the Rcs phosphorelay in an opg-negative strain (as discussed above) resulted in complete loss of virulence on potato tubers (Bouchart et al., 2010). Therefore, it appears that precise fine-tuning of Rcs expression is important for the timing of the production of virulence factors. Interestingly, in P. stewartii, the causative agent of Stewart’s wilt disease in maize, QS finely controls the expression of the accessory regulator RcsA so that production of EPS occurs only after the bacterium successfully colonizes the xylem.
vessels (Burke et al., 2015; Carlier & von Bodman, 2006). Altogether, the different elements of the Rcs phosphorelay may allow the possibility of multiple signals to modulate the regulatory cascade at various stages of the transduction process, and therefore the coordination of gene expression required for precise timing of infection.

**RpfC finely regulates Xanthomonas pathogenesis in a cell density-dependent manner**

Phytopathogens belonging to the genus *Xanthomonas* cause diseases in a wide range of economically important plants. These bacteria rely on cell-to-cell signalling mediated by molecules of the DSF (diffusible signalling factor) family to synchronize virulence gene expression to cell density during plant infection [for a recent review on DSF family cell-to-cell signals, see Ryan et al. (2015)]. The DSF family of signals are cis-2-unsaturated fatty acids, for which the paradigm is cis-11-methyl-dodecenolic acid described from *Xanthomonas campestris* pv. *campestris* (*Xcc*), the causal agent of black rot disease of cruciferous plants (Wang et al., 2004). In *Xcc*, DSF signalling positively controls the expression of virulence factors during infection, such as extracellular enzymes (endoglucanases and proteases) and the EPS xanthan (Ryan & Dow, 2011).

Synthesis and perception of DSF require products of genes within the *rpf* cluster (for regulation of pathogenicity factors).
The synthesis of DSF relies strictly on RpfF, a key enzyme homologous to enoyl-CoA hydratase, and is also partially dependent on RpfB, a putative long-chain fatty acyl-CoA ligase (Barber et al., 1997), whereas DSF perception and signal transduction depends upon the RpfC/RpfG TCS (Slater et al., 2000). RpfC is an HsHK consisting of multi-domains including a transmembrane domain, a transmitter domain, a REC domain and an Hpt domain (Fig. 8a). The cognate RR, RpfG, contains a REC domain attached to an HD-GYP domain, which acts to degrade the intracellular second messenger, bis(3',5')-cyclic diguanosine monophosphate (c-di-GMP) (Ryan et al., 2006, 2010). Comparative genomics studies indicated that the rpf gene cluster is conserved not only in Xanthomonas species but also in bacteria from related genera, such as Xylella fastidiosa (plant pathogen) and Stenotrophomonas maltophilia (some strains are human opportunistic pathogens whereas others can be found in close association with plants) (Lu et al., 2008; Ryan et al., 2011). In the vast majority of these bacteria, DSF positively regulates virulence-associated traits, but in the xylem-limited pathogen X. fastidiosa, DSF-deficient mutants were shown to be hypervirulent on grape but had reduced ability to colonize insect vectors (Chatterjee et al., 2008).

In Xcc, inactivation of rpfF, rpfC or rpfG results in a coordinated reduction in the synthesis of important virulence factors, including extracellular enzymes and xanthan, as well as alteration in biofilm formation, reduction in pilus-dependent motility and a decrease in virulence (Ryan et al., 2006; Slater et al., 2000). Furthermore, addition of exogenous DSF can restore virulence factor synthesis and induce biofilm dispersal in an rpfF-deficient mutant, but not in strains lacking rpfC or rpfG, consistent with the involvement of RpfC/RpfG in sensing and in the transduction of the DSF signal.

**Fig. 8.** RpfC and virulence gene expression in X. campestris. (a) At low cell density, RpfC binds RpfF through its REC domain, thus lowering DSF synthesis (green stars). The unphosphorylated RR RpfG is inactive and c-di-GMP intracellular concentration (red hexagons) remains high in the cell. (b) At high cell density, accumulated extracellular DSF binds RpfC, which induces a conformational change facilitating the release of RpfF and subsequent phosphorelay. Active RpfG acts as a phosphodiesterase and decreases c-di-GMP intracellular concentration, which indirectly promotes extracellular enzyme synthesis. Furthermore, the 'auxiliary' DSF sensor RpfS binds DSF through the N-terminal PAS_4 domain and indirectly influences the expression of genes involved in motility and chemotaxis. Symbols are as follows: purple squares, transmitter domain; blue squares, REC domain; orange squares, Hpt domain; light brown arrow, HD-GYP output domain; green pentagons, RpfF. Black solid and dashed arrows respectively indicate direct and indirect mechanisms. The question mark indicates unknown pathways. Abbreviations: OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane; CP, cytoplasm.
signal (Slater et al., 2000). Nonetheless, knocking out rpfC led to increased DSF synthesis, but not in an rpfG-deficient strain, thus conforming that the HyHK RpfC acts as a sensor for DSF but also represses DSF synthesis (Slater et al., 2000). On the one hand, the DSF ligand binding to the sensory domain of RpfC triggers its autophosphorylation and subsequent phosphorelay, ultimately activating RpfG, which induces the expression of virulence genes by modulating c-di-GMP levels via its HD-GYP domain (He et al., 2006; Ryan et al., 2006) (Fig. 8b). On the other hand, RpfC inhibits DSF production via a post-translational mechanism involving interaction between the RpfC REC domain and RpfF (Cheng et al., 2010; He et al., 2006) (Fig. 8a). Crystal structure resolution of RpfF alone or in complex with the REC domain of RpfC, coupled with mutational analysis, points to a model in which the binding of the RpfC REC domain may block substrate fixation to the active site of RpfF, thereby inhibiting DSF synthesis (Cheng et al., 2010).

This sophisticated regulatory mechanism may constitute an autoregulatory mechanism that allows the DSF signal to self-regulate its biosynthesis.

Recent work aiming at unravelling the overall regulatory influence of rpf components in Xcc pathogenesis has provided evidence for additional complexity of the Rpf/DSF signalling network. Mutation of rpfF, rpfG and rpfC led to alteration of the transcription of approximately 480 genes compared to Xcc WT (An et al., 2013). However, the analysis revealed that RpfF regulates the expression of 48 genes that are not regulated by RpfC, and conversely, RpfC regulates the expression of 135 genes that are not regulated by RpfF. These findings suggest that RpfC may sense other environmental cues (in addition to DSF) and indicates that alternative sensing mechanisms for DSF may exist in Xanthomonas (An et al., 2013). Accordingly, the first PAS domain of a novel DSF-sensing HyHK called RpfS was shown to be involved in sensing

### Table 1. HyHK-based TCS systems highlighted in this review

<table>
<thead>
<tr>
<th>Structure of hybrid HK and cognate RR and species hosting those TCS</th>
<th>Other components of the phosphorelay* or interacting with the phosphorelay†</th>
<th>Cognate signal</th>
<th>Other stimuli affecting the phosphorelay</th>
<th>Regulated phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HyHKRetS†</td>
<td>Unknown signal related to cell density</td>
<td>Nutrient starvation and stress (ppGpp)</td>
<td>Regulatory RNAs→ metabolites and lytic exo-enzymes</td>
<td></td>
</tr>
<tr>
<td>HyHKLadS†</td>
<td></td>
<td>Temperature</td>
<td></td>
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<tr>
<td>HyHKPA1611†</td>
<td></td>
<td>Plant sugars</td>
<td></td>
<td></td>
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<tr>
<td>LysR-type regulator FitG†</td>
<td>Unknown signal from insect</td>
<td></td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ChvE*</td>
<td>Plant phenolic compounds?‡</td>
<td>Monosaccharides</td>
<td>Activation of virulence genes</td>
<td></td>
</tr>
<tr>
<td>Lipoprotein RscF*</td>
<td>Unknown signal related to membrane integrity?</td>
<td>Solid surface</td>
<td>Activation of EPS/ CPS synthesis and PCDWEs</td>
<td></td>
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<tr>
<td>Kinase RcsD†</td>
<td></td>
<td>Osmotic stress</td>
<td></td>
<td></td>
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<td>Regulator RcsA*</td>
<td></td>
<td>Low temperature</td>
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<td>RpfC</td>
<td></td>
<td>Desiccation</td>
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<td>RpfG</td>
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<tr>
<td>DSF synthase RpfF*</td>
<td>DSF</td>
<td>Environmental cues</td>
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<tr>
<td>HyHK RpfS*</td>
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</tbody>
</table>

ND, Not determined.

*Components interacting with the phosphorelay but not participating in phosphotransfer reactions.
†Components that are part of the phosphorelay.
‡Interaction between VirA and phenolic compounds is thought to occur in a region of VirA (the linker region located upstream of the transmitter domain) displaying structural similarity to a GAF domain.
of DSF. Inactivation of RpfS resulted in loss of motility and reduced Xcc virulence in Chinese radish (An et al., 2014) (Fig. 8b). Furthermore, RpfS mutation had a broad effect on Xcc transcriptome, with 424 genes whose expression is significantly influenced, notably those involved in chemotaxis and type IV system secretion (An et al., 2014). Homologues of RpfS are found in many Xanthomonas and Stenotrophomonas strains (but not all), suggesting that it is an accessory sensor involved in DSF signalling (An et al., 2014). In sum, at least two distinct HyHKs sense DSF signals in Xcc and regulate the expression of a distinct subset of genes, allowing fine-tuning of Xanthomonas pathogenesis in the root environment.

Concluding remarks
Successful host–bacteria interaction is the result of an intricate interplay that needs the integration of multiple input signals, i.e. specific environmental and host-driven conditions, as well as the fine-tuning of gene expression. Undoubtedly HyHKs, by providing additional regulatory checkpoints and allowing the fine-tuning of the output response, are key players in the orchestration of plant–bacteria interactions.

The most documented HyHK-based TCS systems reviewed here clearly share little commonality (Table 1). From a structural point of view, HyHKs are all membrane proteins harbouring a transmitter and a REC domain but they vary in length, presence of input and Hpt domains. A few common themes can be tentatively drawn from genetics and structural studies of HyHK: (i) in the absence (or lack of evidence) of an input domain, perception of the signal may be achieved by an accessory protein and transmitted to the HyHK, like RcsF in the Rsc phosphorelay; (ii) the absence of an Hpt domain within an HyHK may indicate that another component of the phosphorelay is needed (like RcsD in the Rcs phosphorelay); however, this might indicate that the REC domain may not serve for phosphate transfer but may fulfill another regulatory role, like VirA of A. tumefaciens; (iii) the internal REC domain can have a dual function: a pivotal role in the signal transduction cascade and a regulatory role, as illustrated with RpfC of X. campestris; and (iv) orphan HyHKs are likely to act through a mechanism that is independent of phosphotransfer activity by interacting with other TCS systems, thus forming multi-sensory signalling networks (such as RetS and LadS of Pseudomonas).

Interestingly, components of some HyHK-based TCS are well conserved, such as the Gac/Rsm system among Pseudomonas spp. and the Rcs phosphorelay throughout the Enterobacteriaceae family (Table 1). Thus, bacteria with contrasting lifestyles (commensal, animal pathogen, plant pathogen) can share common signalling pathways and likely cognate signals but differ in the target genes of these phosphorelays, as shown above with the various Rcs regulons.

As for classical TCS, the phosphorylation cascade of HyHK-based TCS is affected by various environmental stimuli (other than the cognate signal) (Table 1) and is interconnected with other global regulatory networks, including QS, c-di-GMP and small RNAs, to control bacterial physiology in the rhizosphere.

Despite insights into HyHK signalling properties, the recent advances outlined above pose a new set of outstanding questions. As most bacteria encode dozens of HyHKs in their genomes, the challenge ahead is to produce a complete picture of bacterial signalling networks at play during plant interaction. Deciphering the role of the tethered REC domain(s) (expected role in phosphate transfer, other regulatory role or dual function) appears as a key issue in order to highlight the advantages of HyHK-mediated regulation on bacterial physiology. Orphan HyHKs that can represent a high proportion in the genomes of some plant-associated bacteria (Borland et al., 2015) are certainly key players to focus on, aiming to pinpoint the regulation pathways they belong to. Soluble HyHKs that may sense intracellular cues or membrane-diffusible signals have also received very little attention so far. Furthermore, as for all TCS, elucidation of the nature of the signals perceived and the downstream targets remains an important outcome. Future research to address these issues will most likely uncover further fascinating aspects of this family of signalling proteins, and holds great promise for the development of novel strategies to enhance or inhibit plant interaction with these agronomically important micro-organisms.

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