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Regulation of bacterial virulence by two-component systems

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In bacteria, two-component systems (TCS) are widely used signal transduction devices which are engaged in a multitude of gene regulatory systems that respond to changing growth conditions. Many pathogenic bacteria encounter different microenvironments during their infectious cycle and their ability to efficiently adapt to different niches inside and outside of their host organisms is frequently mediated by TCSs, which can, therefore, be considered as an essential prerequisite for their pathogenicity. Although significant progress has been made in the elucidation of basic principles of the signal transduction process itself, in many pathogens the contribution of TCS to bacterial virulence is insufficiently recognized.

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Introduction

Two-component systems (TCSs) are widespread signal transduction devices in prokaryotes that enable these organisms to elicit an adaptive response to environmental stimuli mainly, through changes in gene expression. TCSs were also detected in several eukaryotes including plants, yeasts, fungi and protozoa, although to a much lower extent. Currently more than 4000 TCSs have been identified in 145 sequenced bacterial genomes [1], demonstrating the enormous impact of these systems on environmental adaptation of bacteria. A significant relationship between the number of TCSs and the genome size was observed, with larger genomes tending to encode more TCSs. Similarly, environmental bacteria with a broad metabolic versatility were found to code for more TCSs than did microorganisms living in a uniform habitat. This is particularly prominent in bacteria that have adapted to a particular niche within an animal or human host organism; examples include pathogenic and symbiotic obligate intracellular bacteria such as *Chlamydia*, *Rickettsia*, *Buchnera* and *Blochmannia*, and extracellular pathogens including *Helicobacter pylori* or *Mycoplasma* spp., the latter lacking TCSs entirely.

The TCSs are typically composed of a membrane-located sensor with histidine kinase activity and a cytoplasmic transcriptional regulator. Generally, stimuli detected by these systems are transformed into a cellular signal by autophosphorylation of the sensor proteins at a conserved histidine residue. The phosphorylated histidine of these sensor proteins is the source for phosphorylation of an aspartic acid residue in the so-called 'receiver domain' of the transcription factor. Phosphorylation of the regulatory proteins induces a conformational change which alters their DNA-binding properties. A small number of TCSs are characterized by a complex phosphorelay between two histidine and two aspartic acid residues present in four signalling domains, which can either be independent proteins or be integrated into multidomain TCS proteins in various combinations [2].

Two-component systems and bacterial virulence

Although the basic biochemistry of TCSs is quite well understood, and some structural insights in the phosphorylation-dependent conformational changes of TCS domains and their interactions are available (for reviews see [2,3]), several important issues are less clear. This is particularly true regarding the nature of the environmental cue sensed by the TCS, which has been verified experimentally in very few cases. Among the presumptive signals that are thought to be detected by the TCS are chemical and physical parameters such as different ions, temperature, pH, oxygen pressure, osmolarity, autoinducer compounds, the redox state of electron carriers, and the contact with host cells. Moreover, in many cases the role of the TCSs in the pathogenicity of bacteria is poorly understood, and an attenuation of the virulence properties was described in TCS mutants without a complete understanding of the mechanisms underlying the attenuation (Table 1). The attenuated phenotype of TCS mutants is frequently caused by interference with the cells' metabolic requirements rather than with changes in the expression of specific virulence factors. Investigation of the interplay between bacterial and host metabolism has so far been neglected, although it is key to further understanding the principles underlying the successful infection of a host by a pathogenic microorganism.

There are only a few examples in which an extensive view of mechanisms of virulence regulation by TCSs is currently available. Two-component signalling in bacterial

Table 1

TCSs contributing to bacterial virulence regulation

Organism	TCS	Presumptive stimulus	Regulation of, or effect of inactivation	Reference
S. enterica	PhoP-PhoQ	Mg^{2+}/Ca^{2+}	Mg^{2+} uptake, modification of LPS, resistance to antimicrobial peptides, *pmrD*, transcriptional regulator genes *ssrB*, *hilA*, *slyA*, other virulence related genes post-transcriptional regulation of SsrA	[8,22]
	PmrA-PmrB	Fe^{3+}	Lipid A modification	[58]
	RcsC-YojN-RcsB	Desiccation, osmotic shock, growth on solid surfaces; specific *in vivo* stimulus unknown	Colonic acid capsule synthesis, *ftsA*, *osmC*, motility and chemotaxis genes, *fhlDC*, *tviA*, *rprA*	[15]
	OmpR-EnvZ	Osmolarity	Porin genes, *ssrB-ssrA*, stationary phase acid response	[23,59]
	SsrB-SsrA	ND	SPI-2 TTSS and effector genes	[60]
	SirA-BarA	ND	*csrB*, *hilD*	[27,28]
Shigella flexneri	OmpR-EnvZ		Invasion genes	[61]
S. sonnei	CpxR-CpxA	pH?	Virulence regulator gene *virF*	[62]
Vibrio cholerae	ArcA-ArcB		Virulence regulator gene *toxT*	[63]
Helicobacter pylori	FlgR-FlgS	ND	Flagellar genes	[64]
	ArsR-ArsS	Low pH	Urease and other acid-resistance genes	[65]
Campylobacter jejuni	DccR-DccS	ND	Colonization defect	[66]
Legionella pneumophila	CpxR-CpxA	ND	*icmR* and other *icm-dot* genes, no effect on intracellular replication in amoeba and human macrophages	[67]
	LetA-LetS	ND	Growth defect in amoeba, but not in human macrophages	[68]
Yersinia pseudo-tuberculosis	PhoP	ND	Virulence attenuation, reduced survival in macrophages	[69]
Pseudomonas aeruginosa	AlgR-FimS	ND	Alginate biosynthesis, twitching motility	[70]
	AlgB-KinB	ND	Alginate biosynthesis	[71]
	RocA1-RocS1 (SadR-SadS)	ND	Fimbrial genes, biofilm maturation	[72,73]
	PprB-PrpA	ND	Virulence genes and cell motility, QS signal production	[74]
	RtsM (RetS)	ND	TTSS and effector genes	[75,76]
Brucella abortus	BvrR-BvrS	ND	*omp* genes, virulence attenuation, reduced invasiveness in macrophages and HeLa cells	[77,78]
Neisseria meningitidis	MisR-MisS	ND	Composition of LOS inner core	[79]
B. pertussis	BvgA-BvgS	Temperature, redox state of quinones, SO_4^{2-}, nicotinic acid	Toxin and adhesin expression, biofilm formation	[35,80]
Listeria monocytogenes	DegU	ND	Virulence attenuation	[81]
	VirR-VirS	ND	Virulence attenuation	[82]
	AgrA-AgrC	ND	Virulence attenuation	[83]
	LisR-LisK	ND	Virulence attenuation	[84]
Mycobacterium tuberculosis	DevR-DevS	ND	Virulence attenuation	[85]
	MprA-MprB	ND	Virulence attenuation	[86]
	RegX3-SenX3	ND	Virulence attenuation	[87]
	PrrA-PrrB	ND	Intracellular growth defect during the early stages of macrophage infection	[88]
Streptococcus pneumoniae	CiaR-CiaH	ND	Virulence relevant gene *htrA*	[89]
	RR04-HK04	ND	Virulence genes *psaB*, *psaC*, *psaA*	[90]
	RR06-HK06	ND	Virulence gene *cbpA*	[91]
	RitR	ND	Iron homeostasis	[92]
	MicA-MicB	Oxygen?	Virulence attenuation	[93]
Streptococcus pyogenes	CsrR-CsrS (CovR-CovS)	Mg^{2+}	Capsule synthesis, virulence genes *ska*, *sagA*	[94,95]
Streptococcus agalactiae	CsrR-CsrS (CovR-CovS)	ND	Virulence attenuation	[96,97]
S. mutans	SMRR11-SMHK11	ND	Biofilm formation and acid resistance	[98]
Staphylococcus aureus	AgrA-AgrC	AIP	Regulatory RNA III	reviewed in [4]
	SrrA-SsrB	Oxygen?	Exoprotein genes, RNA III	[99]
	SaeR-SaeS	ND	Exoprotein genes	[100]
	ArlR-ArlS	ND	Exoprotein genes	[101]
	LytR-LytS	ND	Holin-like genes *lrgA*, *lrgB*	[102]
Clostridium perfringens	VirR-VirS	ND	Toxin (*pfoA*, *cpb2*) and adhesion genes (*cna*)	[103]

ND, not determined.

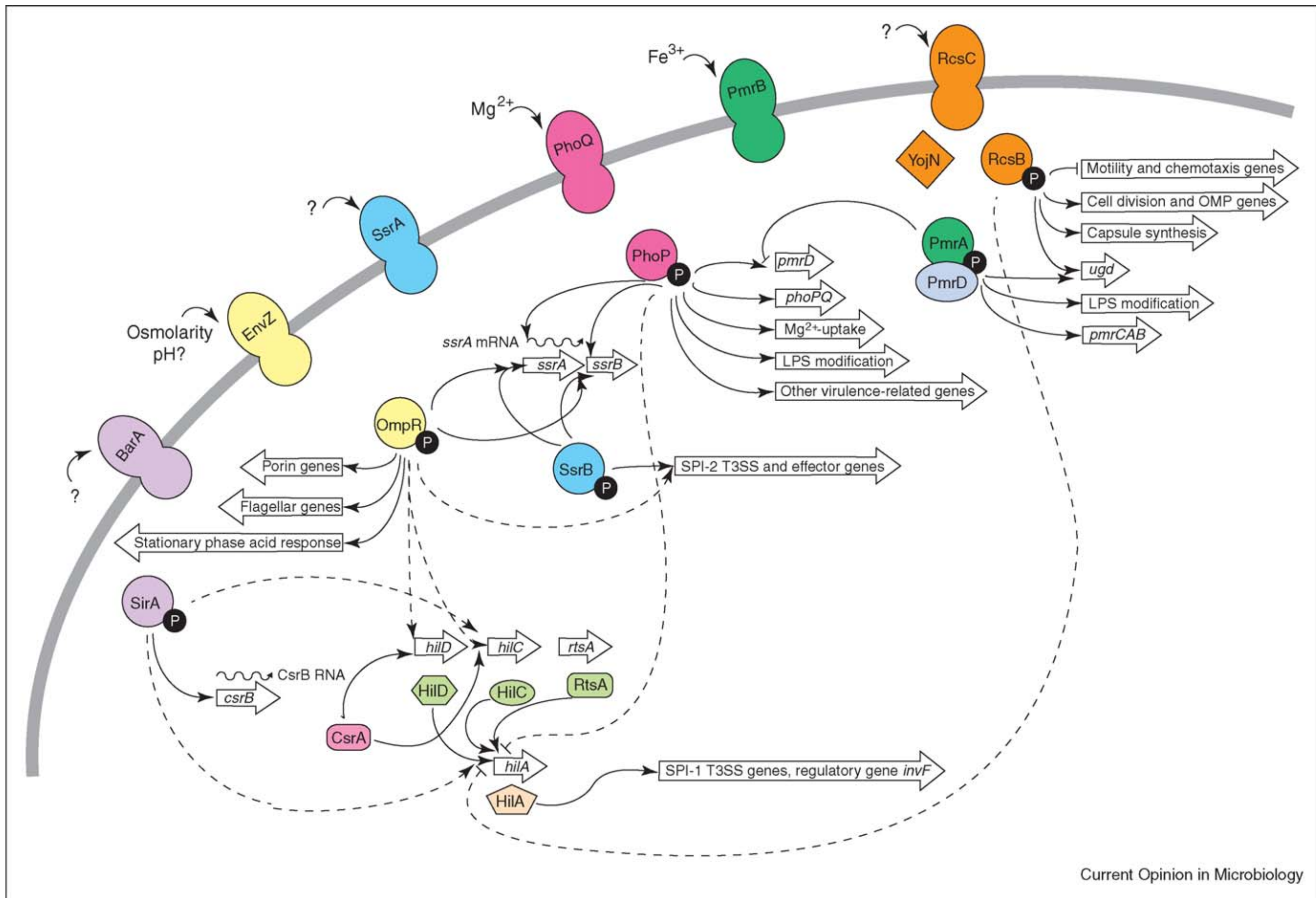
virulence gene regulation exhibits different levels of complexity when integrating various systems into regulatory networks. The regulation systems for *Salmonella* and *Staphylococcus aureus* virulence properties are well characterized, and involve a sophisticated interaction of several TCSs and additional regulators to control expression of virulence factors at different stages during infection. The regulation of *S. aureus* virulence, involving the AgrA-AgrC TCS, which responds to cell-density and controls the transcription of the regulatory RNA III, as well as three additional TCSs named SaeR-SaeS, SsrA-SsrB and ArlR-ArlS, has been reviewed recently [4,5]. The BvgA-BvgS TCS is an intriguing example of a system that appears to be the master regulator of virulence controlling virtually all known virulence traits of *Bordetella pertussis* (see below). Here, we focus on *S. enterica* and *B. pertussis* as paradigm systems for complex TCS-mediated regulatory networks and for a single TCS acting as a general servant, respectively.

The virulence regulatory network of *S. enterica*

S. enterica can cause diseases ranging from self-limiting gastroenteritis to frequently fatal typhoid fever. Many of the virulence traits of *S. enterica* can be attributed to the presence of *Salmonella* pathogenicity islands (SPIs) which, in the case of SPI–1 and SPI–2, encode type III secretion systems conferring to *S. enterica* the ability to actively invade non-phagocytic cells and to replicate within the phagosome (reviewed in [6,7]). The virulence traits of *S. enterica* are controlled by a complex interplay of transcriptional regulators, which involves a sophisticated network of inter-communicating TCSs (Figure 1). The PhoP-PhoQ TCS has long been known as the master regulator of *Salmonella* virulence; its inactivation results in strong virulence attenuation in mice, the inability to survive within macrophages and increased susceptibility to killing by antimicrobial peptides [8]. Significant progress has been made in recent years to unravel the intricate regulatory networks governed by PhoP-PhoQ. The periplasmic concentration of Mg^{2+} and Ca^{2+} ions has been identified as the signal detected by the sensor kinase PhoQ and because the ionic concentration is low in the phagosome, Mg^{2+} is considered the major environmental cue to *Salmonella* from inside the phagocytic vacuole (reviewed in [8]). Recently, the presence of sub-lethal concentrations of cationic antimicrobial peptides was suggested as an additional signal detected by PhoQ, as the exposure of *Salmonella* to polymyxin induced the expression of members of the PhoP-PhoQ regulon, including the autoregulatory PhoP protein, in a PhoP-dependent manner [9]. The PhoP-PhoQ regulon comprises more than 40 genes that can be classified as ancestral genes (i.e. present in other enterobacteria) or *Salmonella*-specific genes, which were presumably incorporated into the *Salmonella* chromosome by horizontal gene transfer. The ancestral PhoP-regulated genes are mainly involved in the uptake of Mg^{2+} and in reducing the Mg^{2+} requirement of the cell envelope. The *pmrD* gene, which mediates the PhoP- and Mg^{2+}-dependent regulation of the PmrA-PmrB regulon that controls genes involved in polymyxin B resistance by modification of the overall negative charge of the lipopolysaccharide, also belongs to this class [10–12]. The PmrA-PmrB TCS is directly activated by the binding of Fe^{3+} ions to the periplasmic domain of PmrB, which results in the inhibition of the PmrA∼P-specific phosphatase activity of the continously autophosphorylating sensor protein. Activation of the PmrA-PmrB regulon by low Mg^{2+} was recently demonstrated to be a result of a specific interaction of PmrD with the N-terminal domain of PmrA∼P, which protects the phosphorylated response regulator from dephosphorylation by its cognate kinase [13•]. Negative feedback inhibition of *pmrD* transcription by PmrA adds further complexity to the system [14]. One target gene of PmrA, *ugd*, encoding UDP-glucose dehydrogenase, which is required both for colonic acid capsule synthesis and lipid A modification, is also controlled by the RcsC–YojN–RcsB phosphorelay system in response to artificial cell-envelope-modifying conditions. Depending on the activating condition, RcsB-dependent transcription of *ugd* requires either RcsA or PhoP as coregulator [15,16]. The specific stimulus activating the RcsC-YojN-RcsB system is unknown. Interestingly, it was observed that permanent activation of the RcsC-YojN-RcsB phosphorelay system by a constitutive mutation in the *rcsC* sensor gene strongly attenuated *Salmonella* virulence in mice by rendering the bacteria unable to invade non-phagocytic cells and to survive within macrophages [17•], suggesting that switching off the expression of genes which are incompatible with the pathogenic lifestyle is of crucial importance for *Salmonella* virulence. Transcription of several invasion genes, including *hilA*, which encodes the major transcriptional regulator of the SPI-1 genes, was abolished in the constitutive *rcsC* mutant [17•]. Recently, the IgaA protein was identified as a post-translational regulator of the activity of the RcsC-YojN-RcsB system [18•].

The *Salmonella*-specific members of the PhoP-PhoQ regulon are largely involved in virulence and intra-macrophage survival. Several of these genes were shown to be regulated by both PhoP and SlyA, the latter being a transcriptional regulator of the MarR family [19,20]. Positive transcriptional control of the *slyA* gene by PhoP has also been reported; however, different studies produced conflicting results [19–21]. A direct link between the PhoP-PhoQ system and intramacrophage survival has been unravelled by Bijlsma and Groisman [22•], who showed that expression of the TCS SsrB-SsrA which regulates the transcription of the *spi* and *ssa* genes encoding the type III secretion system of SPI-2 and of genes encoding Spi and SsA effector proteins, is controlled by PhoP-PhoQ. Control of *ssrB* transcription occurs through direct binding of PhoP to the *ssrB* promoter, whereas

Figure 1



Schematic representation of the regulatory network controlling virulence gene expression in *S. enterica*. The grey line represents the cytoplasmic membrane containing the different histidine kinases depicted in various colours. Cognate response regulators are shown in the same colour as the corresponding histidine kinase. Large arrows indicate the genes or cellular responses controlled by TCSs. 'P' indicates phosphorylation of the respective response regulator. Dashed arrows indicate presumptive regulatory interactions for which conflicting results have been reported in the literature. Besides regulating other virulence genes, the PhoP-PhoQ TCS regulates the expression of the SsrB-SsrA TCS, which controls the transcription of genes encoding the SPI-2 T3SS (type III secretion system) and the respective effector proteins. PhoP-PhoQ is also implicated in the repression of invasion genes encoded on SPI-1. Through PmrD, PhoP-PhoQ affects the transcription of the Fe^{3+}-responsive PmrA-PmrB regulon. OmpR-EnvZ and SirA-BarA are involved in expression control of HilA, the transcriptional regulator of SPI-1 genes. OmpR-EnvZ also contributes to the transcriptional control of *ssrA-ssrB*. See text for details. Abbreviations: OMP, outer membrane protein; LPS, lipopolysaccharide.

expression of SsrA is modulated by PhoP at the post-transcriptional level, an effect that is dependant on the 5′ untranslated region of the *ssrA* transcript [22•]. Interestingly, transcription of *ssrB*-*ssrA* inside macrophages also requires the OmpR-EnvZ TCS, and binding of OmpR∼P to the upstream regions of the *ssrA* and *ssrB* genes has been demonstrated [23]. Furthermore, SsrB∼P was shown to footprint regions located downstream of the transcriptional start sites of *ssrA* and *ssrB* and overlapping the OmpR binding sites, suggesting an autoregulatory role of this response regulator [24]. From these observations Bijlsma and Groisman [22•] hypothesized that OmpR-EnvZ promotes transcription of *ssrB* shortly after the internalization of *Salmonella* by macrophages, whereas at later stages of infection, when the conditions within the *Salmonella*-containing vacuole have changed, the activity of OmpR-EnvZ might decrease and the PhoP-PhoQ system might become responsible for the synthesis of SsrB and SsrA proteins.

Besides regulating the expression of the SPI-2 functions, PhoP is implicated in the repression of invasion genes by negatively regulating the *hilA* gene [25]. The SirA-BarA TCS also helps control the expression of invasion genes. The regulatory effect of SirA-BarA is mediated mainly by the CsrAB system through the regulation of the AraC-like transcriptional regulator HilD [26], which, together with HilC and RtsA, controls the transcription of *hilA*. The CsrA protein, the activity of which is controlled by interaction with the regulatory RNAs CsrB and CsrC,

affects the expression of both HilD and HilC [27], and SirA in turn was shown to induce the transcription of the regulatory RNA CsrB [28]. However, direct binding of SirA to the upstream regions of *hilA* and *hilC* has also been reported [29]. The stimulus that activates the SirA-BarA TCS is unknown; however, it has been suggested that acetyl phosphate might be relevant as a phosphate donor for SirA *in vivo* [28].

Mutations in *opmR-envZ* reduce expression of *hilA* [30] and it was suggested recently that OmpR-EnvZ controls invasion genes by inducing *hilA* transcription through HilD [26]; however, effects of OmpR on the expression or activity of HilC have also been discussed [30].

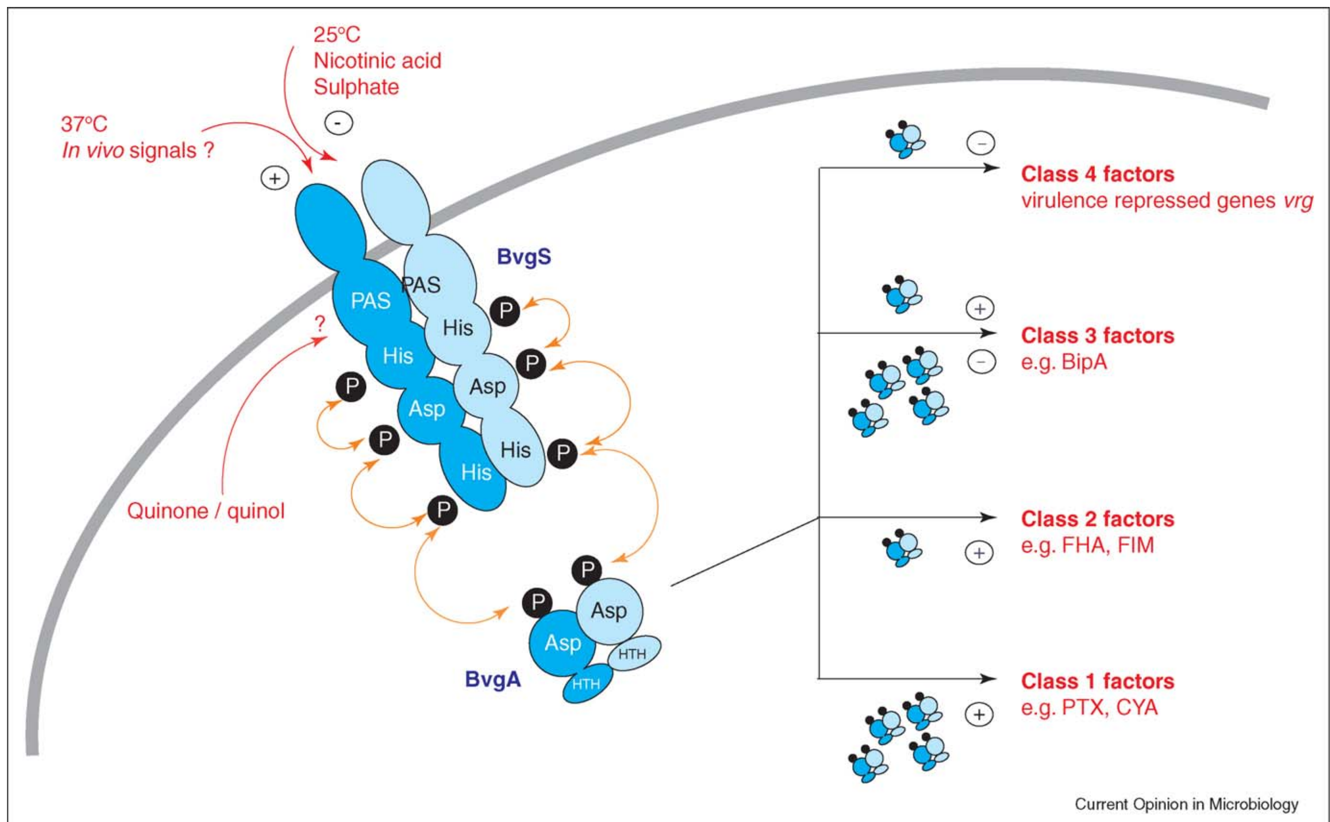
The BvgA-BvgS phosphorelay system of *B. pertussis*

In contrast to *S. enterica*, in which sophisticated regulatory hierarchies mediated by complex interactions between different TCSs and additional regulators, including regulatory RNAs, are involved in virulence gene control, the regulation of virulence genes in *B. pertussis* appears straightforward. In the etiological agent of whooping cough, *B. pertussis*, a single TCS (BvgA-BvgS) appears to be the dominant regulatory system and several surveys to identify auxiliary regulators involved in the differential regulation of individual virulence factors in addition to BvgA-BvgS failed to do so. A prominent feature of the BvgA-BvgS TCS of *B. pertussis* is that it belongs to the family of complex phosphorelay TCSs [31,32]. In the sensory BvgS histidine kinase, a multistep His-Asp-His phosphorelay occurs between different BvgS domains before the transphosphorylation of the BvgA response regulator (Figure 2). Moreover, the BvgS histidine kinase contains a PAS domain of unknown relevance [33]. *In vitro*, the BvgA-BvgS TCS is known to promote virulence gene expression at body temperature and in the absence of certain modulating compounds (e.g. sulphate ions and nicotinic acid). This virulent phase is switched off at low temperature or in the presence of higher concentrations of the modulating compounds, a phenomenon termed 'phenotypic modulation' [34]. Still, it is unclear which signals are relevant during infection, although temperature might be important, as there are significant temperature differences between locations in the nasal cavity and those in the trachea of humans, where BvgS mediated modulation might occur [35]. Moreover, the redox state of quinone electron carriers of the respiratory chain was recently shown to affect BvgS activity [33]. Intracellular signals might therefore also contribute to fine regulation of virulence gene expression.

Class 1, 2, 3 and 4 genes

Detailed analysis of the phenotypic modulation of the BvgA-BvgS system in the recent years has revealed that this system is not just an 'on' and 'off' switch for virulence factor expression, but that the factors controlled by the BvgA-BvgS system can be classified into at least four categories according to their expression pattern and their respective kinetics of transcriptional induction [35]. The so-called class 1 (or 'late') genes include those encoding pertussis toxin (PTX) and adenylate cyclase toxin (CYA, also known as AC or CyaA). Expression of these genes is characterized by the requirement of high concentrations of BvgA~P homodimers which interact with primary binding sites with a relatively low affinity for BvgA~P, and which are located far upstream of the RNA polymerase binding site (−168 bp for *ptx* and −139 bp for *cya*). Cooperative binding of further BvgA~P homodimers leads to interaction of BvgA~P with RNA polymerase and transcriptional activation of the toxin promoters [36,37]. By contrast, the promoters of class 2 (or 'early') genes are characterized by high-affinity binding sites for BvgA~P. Class 2 factors include adhesins such as filamentous hemagglutinin (FHA), fimbriae (FIM) and the autoregulated BvgA-BvgS system. Only relatively small amounts of BvgA~P are required for activation of these promoters. In the case of the *fha* promoter binding of BvgA~P homodimers first occurs at a high-affinity binding site centred around position −88.5 relative to the transcriptional start site. Binding of a BvgA~P homodimer is followed by cooperative binding of two additional BvgA~P homodimers, with the third dimer binding within the −35 box. Interestingly, BvgA~P and the C-terminal domain of the RNA polymerase α-subunit simultaneously interact with the same DNA segment within the promoter, but on different sides of the DNA helix resulting in transcriptional activation [38,39]. Although already anticipated by Lacey's [34] pioneering work on phenotypic modulation in *B. pertussis*, class 3 (or 'intermediate') factors were only identified recently. Among these factors is a gene encoding a protein (BipA) with significant homology to intimin of enterohemorrhagic *Escherichia coli* strains [40]. The regulation of expression of *bipA* involves high affinity binding sites for BvgA~P upstream of the RNA polymerase binding site and low-affinity binding sites within the transcribed region of the respective gene. Binding of BvgA~P at these downstream low-affinity binding sites counteracts the activating properties of the upstream binding sites and causes a decrease of transcription at high BvgA~P concentrations [41,42]. Finally, the BvgA-BvgS system negatively controls the expression of another subset of genes, the so-called class 4 (or 'virulence-repressed' *vrg* genes), the functions of which are not well understood. They are, however, believed to be relevant for survival under starvation conditions and outside of the host, at least in the closely related species *Bordetella bronchiseptica*. *B. bronchiseptica* is an animal pathogen, which, in contrast to *B. pertussis*, has a significant survival capacity outside of the host [43,44]. Mutants locked in this phase by mutations in the BvgA-BvgS system are avirulent. How the negative regulation is mediated by the BvgA-BvgS system is not well understood and a direct interaction of

Figure 2



Schematic representation of BvgA-BvgS mediated virulence gene control in *B. pertussis*. BvgS and BvgA are homodimers. Phosphorylation within the homodimeric BvgS sensor-kinase very likely occurs *in trans* between domains of different monomers, which is not shown in the figure. On the right hand side of the figure the effects of varying BvgA∼P concentrations on the expression of different classes of genes are shown. '**+**' indicates activation and '**—**' indicates repression of expression of the respective factors. Note that BvgA-BvgS both activates and represses class 3 factors, depending on its concentration; low concentrations of Bvg activate class 3 factors, whereas high concentrations repress these same factors.

BvgA∼P at repressor binding sites is possible, as proposed for the *frlAB* locus in *B. bronchiseptica* that encodes a transcriptional activator for flagellar biosynthesis. Alternatively, BvgA∼P might activate transcription of the *bvgR* gene that encodes a presumptive repressor protein [45,46]. Therefore, in the absence of BvgA∼P, only class 4 factors are transcribed. Low amounts of BvgA∼P enable the transcription of the class 2 and 3 factors endowed with high-affinity BvgA∼P binding sites. At full activity of BvgS class 1 and 2 factors are maximally expressed but class 3 factors are again repressed.

Control of the BvgA-BvgS phosphorelay

As mentioned above, these data show that the control of expression of BvgA-BvgS-regulated factors is subtle and depends on the phosphorylation state of the BvgA protein, which can interact in sophisticated ways with its target promoters, as described above. For these reasons, the BvgA-BvgS system was proposed to act more like a rheostat rather than a simple switch. Accordingly, the key to understanding the function of this sensor protein is knowing how the phosphorylation state of BvgA is controlled. In a recent review by Cotter and Jones [35], it was proposed that the particular domain architecture of the BvgS sensory protein and the presence of several mechanisms controlling the phosphate flow in this TCS are crucial for the expression of the various phases. It was previously shown [47] that phosphorylation of the receiver domain of BvgA is mediated by the C-terminal HPt (histidine containing phosphotransfer) domain of BvgS which is phosphorylated at His1172. Phosphorylation of His1172 is mediated by the BvgS receiver, which transfers the phosphate from His729 in the BvgS transmitter to His1172 in the BvgS HPt domain. In addition to this phosphotransferase activity, the BvgS receiver can also act as a phosphatase for both phosphorylated His residues in the transmitter and the HPt domain by catalysing the transfer of the phosphate to a water molecule [47]. Although not experimentally shown, but in analogy to other complex phosphorelay systems such as ArcA-ArcB of *E. coli* [48], it is likely that the phosphotransfer between the HPt domain and the receiver of BvgA is

reversible, which then would allow the BvgS receiver to also act indirectly as a phosphatase for BvgA~P. This scenario implies that the shift between phosphotransferase and phosphatase activities of the BvgS receiver could play a central role in the fine-tuning of the phosphorylation state of BvgA and therefore in the regulation of virulence gene expression. It is still unclear how the opposing activities of the BvgS receiver might be controlled, but it has been proposed that different intensities of stimuli might lead to different BvgS activity states, on the basis of BvgS being active as a dimer, and the combination of different activation states of single domains within the sensor complex allowing the occurrence of various activity states of the BvgS receiver, thereby resulting in fine-tuned control of BvgA phosphorylation [35].

The *in vivo* relevance of these sophisticated fine-tuning mechanisms exerted by a single TCS at different virulence promoters is unclear. The conclusions from previous infection experiments in animal models that have used *Bordetella* mutants carrying mutations in the *bvgS* gene, which lock the bacteria in different expression states or lead to ectopical expression patterns of BvgA-BvgS-regulated factors, are difficult to extrapolate to human infections. Although partially controversial, the available data suggest that minor differences in virulence factor expression are relevant during infection, when *B. pertussis* might encounter relatively mild environmental differences in the various host niches, requiring an extremely fine-tuned adaptation of bacteria to such niches [35,45,49–52]. From these findings, it has been suggested that the expression of different classes of virulence genes reflects slight differences in the growth conditions within distinct host niches colonized by the bacteria, such as in the nasal cavity, larynx and trachea. For example, the temperature within the nasal cavity is lower than in the trachea and might, by subtle modulation, favour the expression of class 3 factors, including BipA. It has been suggested that this class 3 expression profile enables the bacteria to be transmitted efficiently to a new host [35]. It is also speculated that the expression of distinct sets of virulence genes follows the time-course of infection, which requires sequential activation of different sets of virulence factors. In this scenario, adhesins are assumed to be relevant at early steps of infection, whereas later steps require toxin expression to subvert host defence mechanisms [53]. Indeed, a similar sequential expression pattern was recently observed *in vivo* using a recombinase-based *in vivo* technology (RIVET) approach with bacteria that contain different virulence promoters fused to a recombinase reporter. In these experiments the infecting bacteria were synchronized to class 4 gene expression before infection into mice, an experimental requirement to be able to uncover the induction kinetics of the various virulence gene promoters [54•]. However, it is unlikely that during real conditions transmitted bacteria are entirely modulated as in the above mentioned RIVET experiment. By contrast, a bacterial population not synchronized in a particular expression state but consisting of a heterogeneous population with a multitude of expression states within the population might be of advantage especially in the early phase of infection directly after their transmission to a new host. Such a diverse infective population might allow the bacteria to mount an immediate and efficient colonization of different habitats within the host by selection of individuals which are adapted to the respective host niches.

Conclusions

Owing to their versatility in sensing diverse intracellular and extracellular signals and their variable modular architecture, TCSs are convenient devices for the regulation of the expression of virulence properties. Despite the detailed knowledge about the phosphorylation-based signal transduction mechanism itself, surprisingly little information is available about the molecular basis for its contribution to bacterial virulence in most pathogen. What is not known, is the nature of infection relevant signals, their mechanisms of perception, the targets of TCS mediated regulation, and the regulatory networks into which the TCSs are integrated to control the expression of such a multifarious phenotype as bacterial virulence. Future research should be aimed at understanding these features because, owing to the absence of TCS in mammals, these systems might be relevant targets for antimicrobial strategies [55–57].

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- • of special interest
- •• of outstanding interest

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