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Two-component signal transduction as potential drug targets in pathogenic bacteria

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Gene clusters contributing to processes such as cell growth and pathogenicity are often controlled by two-component signal transduction systems (TCSs). Specific inhibitors against TCS systems work differently from conventional antibiotics, and developing them into new drugs that are effective against various drug-resistant bacteria may be possible. Furthermore, inhibitors of TCSs that control virulence factors may reduce virulence without killing the pathogenic bacteria. Previous TCS inhibitors targeting the kinase domain of the histidine kinase sensor suffered from poor selectivity. Recent TCS inhibitors, however, target the sensory domains of the sensors blocking the quorum sensing system, or target the essential response regulator. These new targets are introduced, together with several specific TCSs that have the potential to serve as effective drug targets.

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Introduction

Microorganisms are the most changeable and adaptable organisms on earth. Bacteria, in particular, are found in places where animals and plants cannot survive, such as on glaciers and in hydrothermal vents on the ocean floor. Since their appearance on earth, bacteria have evolved a variety of functions (e.g. signal transduction) to respond to environmental changes. To respond appropriately to different environmental changes, bacteria have evolved two-component signal transduction systems (TCS), which are absent in mammals (including human beings). A typical TCS is composed of a sensor kinase (histidine kinase, HK), which is capable of autophosphorylation in response to an environmental signal, and a response regulator (RR) that interacts with the phosphorylated HK (Figure 1) [1]. The histidine kinase, which is phosphorylated on a conserved histidine residue, transfers the phosphoryl group to the aspartic acid of the response regulator. Many phosphorylated RRs bind to the upstream-regulatory region of pathogenic genes and control their expression. The phosphorylated RR is sometimes also dephosphorylated by the HK. The phosphorylation state of the RR inside the cell is determined by the balance between phosphorylation and dephosphorylation, and this in turn controls gene expression.

To respond to a variety of environmental changes, a bacterium possesses multiple TCSs. Each TCS responds to a specific environmental signal, such as pH, nutrient level, redox state, osmotic pressure, quorum signals, and antibiotics. To respond to diverse environmental changes with greater sensitivity, information is also conveyed between different TCSs to form a complex signal transduction network [2,3]. Some TCSs also control gene clusters that contribute to cell growth, virulence, biofilms, quorum sensing, etc. Conventional antibiotics usually directly target the proteins that carry out essential functions, whereas drugs that specifically inhibit TCSs target the upstream regulatory control functions, not the downstream activities. Thus, anti-TCS drugs will work in a manner different from existing drugs, making possible the development of new drugs and pesticides that are effective against various drug-resistant bacteria. In other words, drugs that target a TCS required for growth may serve as new antibiotics that can kill multi-drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) [4,5,6•,7•]. Furthermore, inhibitors of TCSs that control virulence factors, such as biofilms and quorum sensing (QS), could control virulence without killing the pathogenic bacteria [8,9,10••]. TCSs are also present in eukaryotic microorganisms and are involved in pathogenicity. Thus, TCSs in medically important fungal pathogens are also considered as potential drug targets [11], but will be outside the scope of this review. Here, we review the molecular mechanisms of specific TCSs in bacteria that have the potential to serve as effective drug targets. The first portion of the review describes TCSs that are required for bacterial cell growth. The second portion discusses TCSs that are not required for bacterial cell growth but play important roles in virulence. In TCSs where inhibitor molecules have already been identified,

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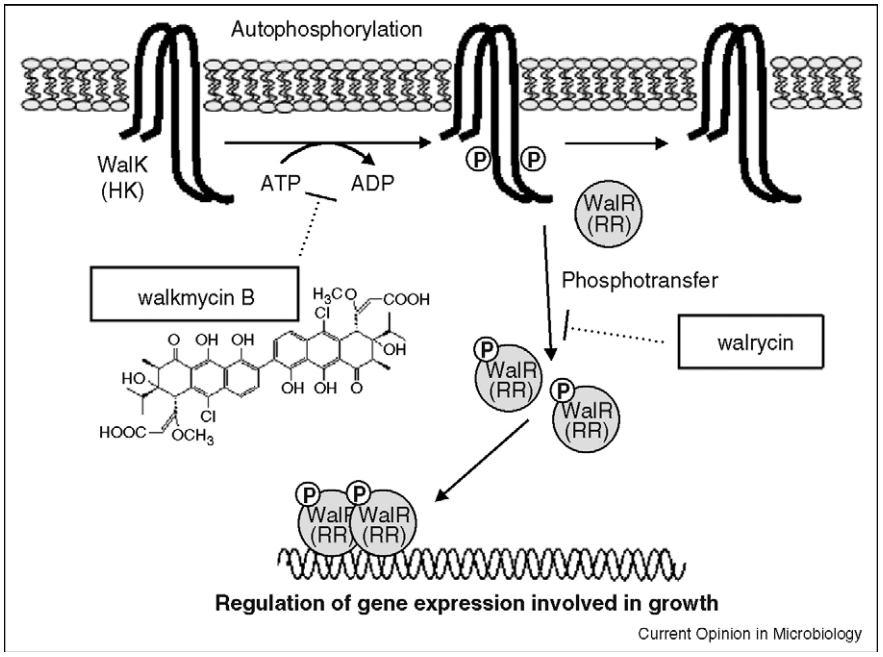
Figure 1



Inhibitors targeting the essential TCS, WalK/WalR. Walkmycin B blocks autophosphorylation of WalK. Walrycin interacts with WalR and blocks phosphotransfer from WalK.

the methods of drug discovery and the identities of the molecules are also discussed.

Essential TCSs

WalK/WalR

WalK/WalR [12–18], YhcS/YhcR81 [19], HP165/HP166 [20], and MtrB/MtrA [21] are essential (Table 1) for growth of bacteria pathogenic to humans. In particular, the WalK/WalR TCS has proven to be indispensable in several Gram-positive species, including *Bacillus subtilis*, *S. aureus*, *E. faecalis*, *Listeria monocytogenes*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus mutans*, and *Streptococcus pyogenes* [22•]. Originally identified in *B. subtilis*, the WalK/WalR system has since been extensively studied, and in these studies it has various designations (YycG/YycF, VicK/VicR, MicA/MicB). Although the function of the WalK/WalR system had remained obscure over the past 10 years, a number of recent reports

Table 1

Essential TCSs for cell growth.

TCS (HK/RR)	Bacterium	Function	Regulated gene	Disease	Ref.
WalK/WalR[a]	*S. aureus*	Cell wall metabolism	*isaA*, *ssaA*, *lytM*	Opportunistic infection	[12]
	S. pneumoniae	Cell wall metabolism	*pcsB*, *lytN*, *fabK*, *pspA*, *piaBCDA*	Pneumoniae	[13]
	S. mutans	Biofilm formation	*gtfBCD*, *ftf*, *gbpB*	Caries	[14]
	S. pyogenes	Cell wall metabolism	Unknown	Necrotizing fasciitis	[15]
	S. epidermidis	Cell wall metabolism	Unknown	Opportunistic infection	[16]
	L. monocytogenes	Unknown	Unknown	Listeriosis	[17]
	E. faecalis	Unknown	Unknown	Urinary tract infection Bacterial endocarditis	[18]
YhcS/YhcR	*S. aureus*	Unknown	Unknown	Opportunistic infection	[19]
HP165/HP166	*H. pylori*	Unknown	Unknown	Chronic gastritis	[20,66]
MtrB/MtrA	*M. tuberculosis*	Unknown	*dnaA*	Tuberculosis	[21,67]

S. aureus: *Staphylococcus aureus*; *S. pneumoniae*: *Streptococcus pneumoniae*; *S. mutans*: *Streptococcus mutans*; *S. pyogenes*: *Streptococcus pyogenes*; *S. epidermidis*: *Staphylococcus epidermidis*; *L. monocytogenes*: *Listeria monocytogenes*; *H. pylori*: *Helicobactor pylori*; *M. tuberculosis*: *Mycobacterium tuberculosis*.

[a] also known as YycG/YycF, VicK/VicR, MicA/MicB.

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revealed that this TCS plays a key role in cell wall metabolism [23], and thus it has lately been referred to as WalK (HK) and WalR (RR) (Figure 1).

In order to isolate inhibitors targeting the WalK/WalR signal transduction pathway, novel drug discovery methods (differential growth assay and homodimerization assay) have been employed [24]. The new methods selectively screen for inhibitors among natural products and a synthetic compound library. By performing a differential growth assay using a temperature-sensitive *walR* mutant (CNM2000) of *B. subtilis*, walkmycin B was isolated as a WalK inhibitor from culture broths of *Streptomyces* sp. [6•]. Moreover, using a homodimerization assay, walrycin B targeting WalR was isolated [7•]. Both walkmycin and walrycin also showed antibacterial activity against MRSA with minimum inhibitory concentrations at 0.20 and 3.13 μg/ml, respectively. These results suggest that WalK and WalR inhibitors are a new class of antibiotics effective against the multi-drug-resistant bacteria MRSA (Figure 1).

Using a structure-based virtual screen of a small molecule lead-compound library, another class of WalK inhibitors active against *S. epidermidis* were discovered, all of which were thiazolidinone derivatives [16]. The inhibitors displayed bactericidal effects on biofilm cells of *S. epidermidis*, indicating that the inhibitors can serve as potential agents against *S. epidermidis* biofilms. Importantly, these inhibitors did not affect the stability of mammalian cells or hemolytic activities.

Non-essential TCSs

Virulence regulated by TCS

Virulence factors of pathogenic bacteria include the production and secretion of toxins as well as the proteases and lipases involved in host invasion. Other important factors include those involved in motility to reach the host, adherence to the host, colonization, and survival. For survival, the bacteria need to adapt to environmental changes upon entry into the host, such as different pH and osmotic pressure, and resist attacks from the host, such as those by antimicrobial peptides. Resistance against antibacterial drugs is also an advantage. Also important for effective colonization and survival are factors involved in biofilm formation or entry into a dormant state such as sporulation. Pathogenic bacteria express these factors as a specific response to a particular environment, and this response, in many cases, is dependent on TCSs. For example, *Pseudomonas aeruginosa*, which inhabits diverse environments, is estimated to have 64 HKs and 72 RRs. Among the TCSs, 19 TCSs are involved in some way with virulence or antibiotic resistance [25]. Even for Gram-positive *S. pneumoniae*, 10 of its 13 TCSs are involved in pathogenicity. In this part of the review, we present the molecular mechanisms of nine TCSs (Table 2) that are potential targets for antivirulence therapy.

QseC/QseB

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is responsible for outbreaks of bloody diarrhea and hemolytic–uremic syndrome. The virulence genes of EHEC (LEE, locus of enterocyte effacement) are rapidly induced when cell density is increased, owing to secretion of chemicals by EHEC (quorum sensing) [26]. The secreted chemical is autoinducer 3 (AI-3). Furthermore, LEE1 gene expression is also induced with the addition of the human hormones epinephrine and norepinephrine [27]. QseC/QseB is the TCS that responds to AI-3, epinephrine, and norepinephrine (Figure 2). In other

Table 2

Non-essential TCSs involved in virulence.

TCS (HK/RR)	Bacterium	Function	Regulated gene	Disease	Ref.
Human pathogens					
QseC/QseB	EHEC	Quorum sensing Regulation of the flagellar regulon	*flhDC*	Bloody diarrhea, HUS	[10••,28••]
		Regulation of the LEE gene	*ler*		
		Shiga toxin	*stx*		
AgrC/AgrA	*S. aureus*	Adhesion, Invasive factor	RNA III	Opportunistic infection	[8,29]
FsrC/FsrA	*E. faecalis*	Protease	*gelE*, *sprE selE-sprE*	Opportunistic infection	[44]
GacS/GacA	*P. aeruginosa*	Lipase, Elastase, Biofilm, Virulence	*rsmY*, *rsmZ*	Opportunistic infection	[25,45••]
PhoQ/PhoP	*S. enterica*	Virulence, Cationic antimicrobial Peptide resistance	*ugd*, *pbgD*	Diarrhea	[49,50]
Plant pathogens					
GacS/GacA	Ecc	Extracellular pectinase Cellulase, Protease, Virulence	*rsmB*	Soft-rot	[47]
PehS/PehR	Ecc	Endopolygalacturonase	*pehA*	Soft-rot	[53]
CorS/CorR	*P. syringae*	Synthesis of coronatine	*cfa*	Chlorosis	[54]
HrpX/HrpY	*E. amylovora*	Type III protein secretion	*hrpL*	Fire blight	[59]

EHEC: Enterhemorrhagic *Ecsherichia coli*; *S. aureus*: *Staphylococcus aureus*; *E. faecalis*: *Enterococcus faecalis*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *S. enterica*: *Salmonella enterica*; *Ecc*: *Erwinia carotovora* spp. *carotovora*; *P. syringae*: *Psudomonas syringae*; *E. amylovora*: *Erwinia amylovora*; HUS: Hemolytic–uremic syndrome.

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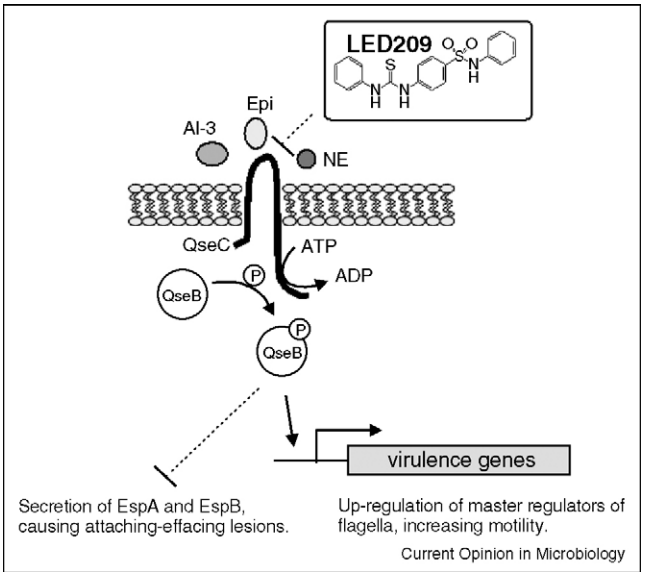
Figure 2



QseC/QseB TCS and its inhibitor. AI-3: auto-inducer 3; Epi: epinephrine; NE: norepinephrine LED209 (*N*-phenyl-4-[[(phenylamino)thioxomethyl]amino]-benzenesulfonamide) blocks QseC autophosphorylation by inhibiting the binding of AI-3, Epi and NE.

words, these signals directly act on the QseC (HK) sensor and promote the autophosphorylation of QseC. The phosphorylated QseC interacts with QseB (RR) and transfers the phosphoryl group to QseB. The phosphorylated QseB binds to the promoter of EHEC's pathogenic genes and controls their expression. Consequently, an inhibitor of the QseC/QseB TCS would suppress only the virulence without killing the EHEC cells.

To search for chemical compounds that inhibit the activation of QseC by AI-3/epinephrine/norepinephrine, a high-throughput screen was carried out using the EHEC strain with a *Lee1-lacZ* reporter in its chromosome and a library of 150 000 compounds [10••]. The compound LED209 inhibits the binding of AI-3, epinephrine, and norepinephrine to QseC. LED209 suppresses EHEC's pathogenic genes *LEE1*, *flhDC*, and *stx2A*, while at the same time inhibiting the autophosphorylation of QseC. LED209 suppresses the pathogenicity of EHEC *in vitro* and *in vivo* but does not inhibit proliferation. Because homologs of QseC exists in at least 25 bacterial species pathogenic to humans or plants, a QseC inhibitor is expected to be a promising drug for antivirulence therapy against a wide range of pathogenic bacteria [28••].

AgrC/AgrA

S. aureus is an opportunistic pathogen, and the rise of MRSA has become a major public health problem. *S. aureus* produces autoinducing cyclic thiolactone peptides (AIPs), which are secreted outside of the cell. The virulence of *S. aureus* is controlled by AIPs and the AgrC/AgrA TCS (*agr* QS system) (Figure 3) [29]. Under the *agr* QS system, signal AIPs are produced when AgrD proteins, which are first produced, pass through the cell membrane and are processed by AgrB, which resides in the cell membrane. The processed AIPs act on the sensor AgrC, which leads to the autophosphorylation of AgrC and the transfer of the phosphoryl group to AgrA [30]. The phosphorylated AgrA directly binds to the DNA region between the RNA III gene and the *agrB* gene, and promotes transcription of RNA III. RNA III downregulates the expression of surface adhesions while upregulating the expression of invasive virulence factors such as proteases, secreted toxins, and lipases [29]. Apolipoprotein B, a major structural protein of lipoproteins, has been reported to inhibit the *agr* QS system by sequestering AIP1. Apolipoprotein B exists in the host's serum and works as an innate defense effector in the host against infections [31]. The AIP–AgrC receptor pair shows considerable interstrain sequence variation. *S. aureus* strains can be divided into at least four *agr* specificity groups. Because non-cognate AIPs work antagonistically against cognate AIPs, it may be that peptides with an amino acid sequence similar to AIPs work as AIP antagonists to inhibit the AgrC/AgrA system [32]. It is suggested that certain non-cognate AIPs stabilize an inhibitory receptor conformation that may be a crucial feature of the ligand–receptor interaction [33]

In addition to the *agr* QS system, a second QS system consisting of an autoinducer RNA III activating protein (RAP) and its target molecule TRAP has also been shown to induce virulence in *S. aureus* [34]. RAP is suggested to regulate the phosphorylation of TRAP, followed by activation of *agr*, but its mechanism remains controversial [35–37]. A heptapeptide RIP (RNA III inhibiting peptide) interferes with this second QS system by competing with RAP, and thus blocking *agr* expression [38]. A nonpeptide analog of RIP, found by virtual screening of a RIP-based pharmacophore against a database of small molecular compounds, also inhibited the expression of RNA III and prevented device-associated infections caused by MRSA strains *in vivo* [39].

FsrC/FsrA

E. faecalis is a Gram-positive intestinal commensal of humans and other animals, but it sometimes causes opportunistic infections [40]. Nosocomial infections caused by multiple-antibiotic-resistant or vancomycin-resistant *E. faecalis* have become a serious clinical problem [41]. The pathogenic genes of such *E. faecalis*, the gelatinase gene (*gelE* encoding a metalloprotease) and *sprE* (encoding a serine protease) [42], are located on the same operon and their genetic expression is dependent on the concentration of GBAP (gelatinase biosynthesis-activating pheromone, a cyclic peptide carrying a lactone

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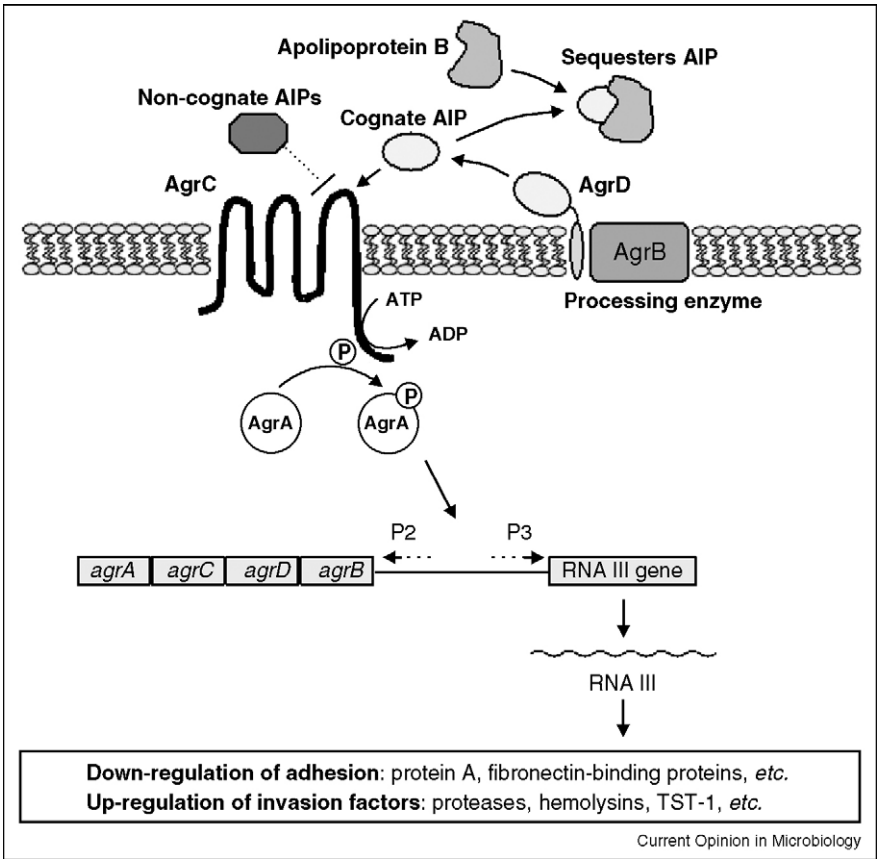
Figure 3



AgrC/AgrA TCS and its inhibitor. Apolipoprotein B inhibits the AgrC/AgrA system by sequestering AIP1. Non-cognate AIPs work antagonistically against cognate AIPs.

ring) secreted outside the cell [43]. In turn, the production and secretion of GBAP is positively controlled by the FsrC/FsrA TCS. GBAP triggers the FsrC/FsrA TCS controlling the expression of *fsrBDC* and *gelE-sprE*, leading to extensive production of gelatinase and serine protease and causing virulence. Therefore, an FsrC/FsrA TCS inhibitor is expected to suppress the virulence of *E. faecalis*. To search for inhibitors of *fsr* QS, compounds that inhibited gelatinase and GBAP production were screened from actinomycetal extracts [44]. A peptide antibiotic, Siamycin I, was identified from the actinomycete culture supernatant selected from the screen. Siamycin I suppressed the transcription of *fsrBDC* and *gelE-sprE*, thus suggesting that it inhibited the GBAP signaling via the FsrC/FsrA TCS.

GacS/GacA

P. aeruginosa is an opportunistic pathogen and the most common cause of acute nosocomial pneumoniae. Such virulence is determined by direct interaction between the sensor histidine kinases GacS and RetS [45••]. In *P. aeruginosa*, GacS/GacA positively controls the transcription of the genes *rsmY* and *rsmZ*, which encode small RNAs. RsmY and RsmZ interact with RsmA (RNA-binding protein) to control a number of virulence factors, for example acyl-homoserine lactones, pyocyanin, lipase, elastase, biofilms, and motility [46]. In the plant pathogen *Erwinia carotovora* subsp. *carotovora*, GacS/GacA mediates the production of *rsmB* (small RNA)-RsmA, which leads to the expression of soft-rot disease [47]

PhoQ/PhoP

Salmonella is a major food-borne pathogen for humans. Among their TCSs, the PhoQ/PhoP system is the major regulator of virulence. The sensor PhoQ detects extracellular Mg^{2+} concentration and is estimated to modify expression of up to 3% of the *Salmonella* genes via its response regulator, PhoP [48]. The PhoQ/PhoP system is considered to regulate *Salmonella*'s abilities to invade epithelial cells, to survive within phagocytic cells, and to resist antimicrobial peptides [49,50]. Since HKs share a unique ATP-binding Bergerat fold with the GHL (gyrase, Hsp90, and MutL) family of proteins [51], interactions of GHL inhibitors with the catalytic domain of PhoQ was examined by NMR chemical shift perturbation. Among the inhibitors, radicicol (an Hsp90 inhibitor) bound and

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interacted specifically with residues in the ATP-binding pocket of PhoQ, as examined by crystallographic approaches. Radicicol inhibited the autokinase activity of PhoQ, however, with a relatively low affinity (10% inhibition by 1 mM radiocol). This suggests that GHL inhibitors may be useful as lead compounds for developing HK inhibitors (in this case, inhibitors against a broad range of HKs, and not only against PhoQ) [52], although sufficient selectivity for HKs compared with other mammalian enzymes with the Bergerat fold is necessary.

The PhoQ/PhoP system is also linked to virulence in *E. carotovora*, where it is called PehS/PehR. The production of endopolygalacturonase and the transcription of *pehA* (endopolygalacturonase gene) are repressed as well as virulence to plants by a deficiency of PehS/PehR [53]. Thus, inhibitors that target PehS/PehR would probably be effective against pathogenic bacteria that use endopolygalacturonase to harm plants.

CorS/CorR

Pseudomonas syringae produces the phytotoxin coronatine (COR), which leads to chlorosis. COR is produced by the coupling of coronamic acid (CMA) and coronafacic acid (CFA). In the CorS/CorR system, CorS senses temperature and activates response regulator CorR through phosphotransfer. The activated CorR binds to the *cfl* promoter region, located at the 5′ end of the CFA biosynthetic gene cluster, and to the promoter region of the CMA biosynthetic gene cluster for transcriptional activation [54,55]. Because CorR deficiency suppresses the production of COR, the virulence factor leading to chlorosis [56], a TCS inhibitor targeting CorS/CorR is expected to be effective against the virulence of *P. syringae*.

HrpX/HrpY

Some plant pathogens cause virulence by secreting plant cell-wall-degrading enzymes (endopolygalacturonase or pectate lyase) or phytotoxin from inside the bacteria. The system involved with secretion is known as the Type III secretion system (T3SS) [57]. The HrpX/HrpY TCS, which detects low pH, low nutrient level, and low temperature, controls the synthesis of *Erwinia amylovora*'s T3SS. The TCS activates *hrpS*, which encodes an enhancer protein. HrpS interacts with a σ^{54} RNA polymerase and initiates the transcription of *hrpL*. HrpL is a sigma factor, and positively controls not only the expression of several genes (*dspE*, *hrpW* and *hrpN*) encoding proteins that are secreted via the T3SS, but also genes (*hrpA*, *hrpC* and *hrpJ*) that encode components of T3SS [58–62]. Mutant strains deficient in *hrpX* and *hrpY* are less virulent than wild type [61]. Research on compounds repressing gene expression of T3SS was carried out for *Erwinia chrysanthemi* [63]. Consequently, *p*-coumaric acid (PCA) suppressed the expression of T3SS related genes. PCA also reduced the promoter activity of *hrpS* and *hrpL*, suggesting its inhibition of the HrpX/Y–HrpS–HrpL regulatory pathway. Whether PCA directly inactivates the HrpX/HrpY system is still not known.

Box 1

- What are the signals for WalK and other essential HK sensors?
- Can TCS inhibitors that target the quorum sensing system be clinically effective?
- Is it possible to discover TCS inhibitors, which act against a broad range of TCSs, but act only on TCSs?
- Will more inhibitors targeting response regulators be found? Are such inhibitors effective against virulence or cell proliferation?
- Will inhibitors targeting other steps of the TCS signal transduction besides autophosphorylation (such as protein–protein interaction) be found?
- Can TCS inhibitors be developed as agricultural chemicals?

Conclusions

Many of the inhibitors introduced in this review target the sensory domain of HKs. However, the signals detected by sensors are still unclear in many of the TCSs involved in virulence. Furthermore, there are many cases where multiple TCSs are involved with one another. To inhibit such TCSs, it may be more effective to target not the sensory domain of sensors, but rather the domains preserved among many TCSs, so as to shut down multiple TCSs at the same time. Previously, many HK inhibitors have been reported, but most of them were compounds with poor selectivity for TCSs and with multiple mechanisms of action, such as membrane damaging properties [64,65]. Continuous efforts are being made (see Box 1) toward the discovery of TCS inhibitors, which act against a broad range of TCSs, but highly specific to TCSs (not inhibiting other kinases). Such inhibitors may be developed into new antibiotics against multi-drug-resistant pathogens, and at the same time, be used as antivirulence agents for pathogens without essential TCSs.

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