Essentiality, Bypass, and Targeting of the YycFG (VicRK) Two-Component Regulatory System in Gram-Positive Bacteria[▽]

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Bacterial two-component systems and phosphorelays are woven into the fabric of cellular regulatory mechanisms ensuring homeostatic equilibrium under a wide variety of environmental conditions (reviewed in references 13, 15, 18, 19, 23, and 28). Of the individual two-component regulatory systems (TCSs), YycFG has excited interest for several reasons and has been the topic of many recent studies. The YycFG system appears to be essential for growth in most bacterial species that encode it (10, 17, 27, 48). The essentiality may be linked to its control of murein biosynthesis (1, 3, 6, 7, 21, 26, 32, 33), cell division (10, 12, 21), lipid integrity (3, 27, 29, 33), exopolysaccharide biosynthesis and biofilm formation (1, 2, 6, 39, 41), and virulence factor expression (2, 6, 24, 26, 33, 39). Because of these effects on essential functions and the fact that the YycFG TCS is widely conserved in low-GC gram-positive bacteria, including several major pathogens, it has been considered a potential target for anti-infective therapeutics (see, e.g., references 14, 25, 35–37, and 42).

Interestingly, the YycFG TCS regulates different sets of genes in different bacterial species to coordinate and control the disparate, yet related, vital functions listed above (3, 6, 29, 33). The signals sensed by the YycFG TCS to maintain cell surface and murein homeostasis are largely unknown; however, the YycFG TCS seems to be one of few TCSs that integrate signals through physiologically relevant cross talk. The best-studied example of cross talk in this system is between YycFG and the PhoPR phosphate limitation TCS in *Bacillus subtilis* (21, 22). In addition, the YycFG TCS includes several auxiliary proteins in its complex regulatory circuits, making it in fact at least a four-component regulatory system in some bacterial species (34, 45, 46). However, recent studies have shown that there are instances where the YycFG TCS appears not to be essential in some bacterial systems (see below) (11, 26, 32). Such results have brought into question the value of TCSs in general and YycFG in particular as therapeutic targets. We argue here that instances of YycFG nonessentiality may be due to genetic bypass mechanisms, and their existence does not diminish the importance of the YycFG TCS in bacterial physiology and pathogenesis or the potential of this TCS and other TCSs from serving as targets for antibiotic development. Furthermore, the real benefit that has emerged from studying the YycFG TCS across species is the realization that this type of TCS may be integrated into higher-order homeo-

static regulatory mechanisms with common goals in all gram-positive species despite the disparate gene targets in each.

The core of the YycFG TCS consists of the YycG histidine kinase and the YycF response regulator (Fig. 1). Because this TCS was discovered independently in different bacterial species, there are several different names for it. However, the YycFG designation from *B. subtilis* has been widely used in many papers for bacterial species other than *Streptococcus*, where YycFG are designated VicRK (Fig. 1). The different names for the YycG and VicK histidine kinases coincide with different structural features (34, 45, 46, 48) (Fig. 1). In non-*Streptococcus* species such as *B. subtilis*, *Staphylococcus aureus*, and *Enterococcus faecalis*, YycG contains a large extracellular domain between two transmembrane domains (10, 16, 27). In contrast, VicK from *Streptococcus*-related species is generally anchored to the cell membrane by a single transmembrane domain (34, 48). Exceptions such as VicK from *Lactococcus lactis* contain two transmembrane domains but still lack an extracellular domain. The YycG and VicK histidine kinases contain similar HAMP- and PAS-sensing domains along with the dimerization/histidine phosphotransfer (HisKA) and kinase catalytic (HATPase) domains found in other histidine kinases (Fig. 1) (reviewed in references 23 and 28). In contrast to the YycG and VicK histidine kinases, the amino acid sequences of the receiver and effector domains of YycF and VicR are highly conserved and belong to the OmpR family of response regulators (reviewed in references 13 and 43).

In most parent strains studied to date, the gene encoding the YycF (VicR) response regulator cannot be simply knocked out and is essential for growth in rich laboratory media. The exceptions to this generalization are strains that likely contain some form of bypass mutation, as discussed below. In contrast, there is again a dichotomy between the YycG and VicK classes of histidine kinases (Fig. 1). The genes encoding the YycG class of histidine kinase are essential and cannot be knocked out. In contrast, the VicK class appears to be dispensable in different species of *Streptococcus* (9, 26, 32, 39, 48). Phosphorylation of the VicR response regulator seems to be required for growth (9, 32). This observation implies that cross talk by other histidine kinases or small phosphoryl group donors, such as acetyl phosphate, phosphorylates VicR in deletion mutants lacking VicK. However, this apparent lack of essentiality of *vicK* can be misinterpreted. The growth properties of $\Delta vicK$ mutants have been studied using a relatively limited number of conditions, and it is possible that other conditions that require VicK for growth will be found. In addition, *vicK* knockout mutants show defects in growth and biofilm formation, increased cell chaining, and reduced virulence in different species of *Streptococcus* (1, 2, 24, 26, 32, 39). Thus, functional

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FIG. 1. Arrangements of genes in the operons encoding the essential YycFG, VicRK, and MtrAB TCSs, domains in the YycG, VicK, and MtrB histidine kinases, and cellular locations of proteins. The operons are drawn to scale from representative species, *Bacillus subtilis* (A), *Streptococcus pneumoniae* (B), and *Streptomyces coelicolor* (C). Essential genes in the species tested to date are surrounded by solid lines, and those that are dispensable are surrounded by dotted lines. Homologous genes are shown in the same color. A sixth gene of unknown function, designated *yycK*, is located downstream of *yycJ* in *Bacillus*, *Listeria*, *Lactobacillus*, and *Oceanobacillus* species but is absent from *Staphylococcus* and *Enterococcus* species. The cytoplasmic domains of YycG and VicK are similar and include HAMP, PAS, HisKA, and HATPase, whereas VicK of *Streptococcus* lacks the second transmembrane and extracellular domain present in YycG. MtrB contains an extracellular domain but lacks a cytoplasmic PAS domain. The extracellular auxiliary proteins YycHI (brown and yellow ovals) and the LpqB lipoprotein (orange oval) are thought to play roles in signaling through the YycG and MtrB histidine kinases, respectively. The functions of the cytoplasmic, conserved YycJ/VicX auxiliary proteins (blue ovals) are currently unknown. The cytoplasmic YycF/VicR and MtrA response regulators are shown as green ovals. See the text for additional details.

YycG and VicK histidine kinases are required for normal cellular physiology and pathogenesis.

It is possible to bypass the essentiality of the VicRK TCS entirely in some bacterial species. In *Streptococcus pneumoniae*, the VicRK TCS positively regulates the transcription of several surface proteins and virulence factors (29, 32, 33). Of this group, only the *pcsB* gene, which encodes a putative hydrolase required for murein biosynthesis and cell division, is essential for growth (31, 32). A synthetic, constitutively expressed promoter fused to *pcsB* bypasses the requirement for positive regulation by the VicRK TCS, and the gene encoding the VicR response regulator can be deleted (32). However, even modest misregulation resulting in approximately a two- to threefold decrease in the amount of PcsB causes significant defects in cell division, susceptibilities to stress conditions, and virulence (31, 32). In contrast to the case in *S. pneumoniae*, the essentiality of the YycF response regulator is more complicated and appears to be polygenic in most other bacteria (3, 6). For example, the coregulation of a group of nonessential, partially redundant genes that mediate murein metabolism is essential for normal cell division and growth of *B. subtilis* (3). In this case, a simple bypassing of YycF function has not been possible to achieve genetically.

There are two other instances in the literature where the YycFG (VicRK) system appears to be nonessential, possibly due to bypass. An insertion of a suicide vector into *vicR* of *Streptococcus pyogenes* (group A *Streptococcus*) could be obtained only at a frequency 3 orders of magnitude lower than that of a control construct (26). Therefore, it seems likely that this mutant contained suppressors that allowed the expression

of the essential gene(s) controlled by VicR. Consistent with this interpretation, clean deletion mutations could not be constructed in *vicR* (26). It is noteworthy that the *vicR* insertion mutant showed defects in growth, viability, and virulence compared to the parent strain (26). Some of these phenotypes were complemented by a wild-type copy of the *vicR* gene, but positive complementation does not necessarily rule out the presence of suppressors and indicates that these phenotypes are due to the misregulation of nonessential genes in the VicRK regulon. A second example of the inactivation of this system was reported for a clinical isolate of *Staphylococcus aureus* following treatment with the lipopeptide antibiotic daptomycin (11). This isolate, which showed increased resistance to daptomycin, contained a single-nucleotide A insertion that caused a frameshift early in *yycG*. However, clinical and laboratory isolates with decreased susceptibility to daptomycin also contained other mutations, such as lesions in *mprF* (lysylphosphatidylglycerol synthetase) (11), and it is possible that one of these other mutations contributes to suppression or cross talk that allows YycG function to be bypassed.

The auxiliary proteins of the YycFG (VicRK) TCS also influence its function and apparent essentiality. In all bacteria that contain this TCS, the genes encoding the histidine kinase and response regulator are cotranscribed with a gene encoding a third component, designated YycJ in *B. subtilis* and most other gram-positive species and VicX in species of *Streptococcus* (Fig. 1). In addition, except in species of *Streptococcus*, the *yycFG* and *yycJ* genes are cotranscribed with two other genes, designated *yycH* and *yycI*, which encode other components of the TCS (Fig. 1) (45–48). Thus, bacteria that contain the YycG

or VicK class of histidine kinases potentially contain a five- or three-component system, respectively (34, 45). The YycJ (VicX), YycH, and YycI proteins seem to be dispensable for growth under the limited number of conditions tested so far. The cytoplasmic YycJ (VicX) protein contains a putative metal binding site in a β -lactamase fold (48), but its catalytic function, if any, is unknown. In *B. subtilis*, YycJ does not seem to be tied to YycFG TCS signaling (45), whereas the VicX protein does seem to play some unspecified role in signaling through the VicRK TCS in species of *Streptococcus* (32, 40). Considerably more is known about the function and structures of the YycH and YycI proteins, which are membrane bound and contain extracellular domains (41–43) (Fig. 1). Knockout mutations of *yycH* and *yycI* were recovered as suppressors that allowed the growth of a temperature-sensitive *yycF* mutant at a raised, nonpermissive temperature (44–46). A current model for this suppression is that YycHI normally negatively regulate the autophosphorylation of the YycG histidine kinase and that the lack of YycHI leads to the increased phosphorylation of the YycF response regulator in the temperature-sensitive mutant (45). Thus, changes in the functions of the YycHI auxiliary proteins may act to bypass some defects in YycF function. However, to date, this form of bypass is not complete and still requires the partially active YycF response regulator. Additional unlinked genes may also play roles in signal transduction by the YycFG (VicRK) TCS. For example, a recent study suggests some direct or indirect link between the function of the VicRK TCS and the StkP serine/threonine kinase of *S. pneumoniae* (38).

A broader biological parallelism between the YycFG (VicRK) TCS and a conserved essential TCS in high-GC gram-positive species, such as the *Actinobacteria*, which include industrially important *Streptomyces* and medically important *Mycobacterium* and *Corynebacterium* species, has emerged. This essential TCS, designated MtrAB (reviewed in reference 20), shares several properties with the YycFG (VicRK) TCSs in low-GC gram-positive species. Similar to the case in *Streptococcus*, the MtrA response regulator is essential for growth in mycobacteria and possibly *Streptomyces*, whereas the MtrB histidine kinase is dispensable (5, 20, 49). This situation again suggests the possibility of physiologically relevant cross talk (20). However, the YycG (VicK) and MtrB histidine kinases are not strict orthologues, since MtrB lacks a PAS domain, which is a conserved feature of YycG (VicK) histidine kinases (Fig. 1). Most intriguingly, the MtrAB TCSs play roles in maintaining normal cell division and envelope synthesis (4, 5, 20, 30, 49), parallel to the functions of the YycFG (VicRK) TCSs in low-GC gram-positive species. Even in corynebacteria, where the *mtrAB* TCS genes can apparently both be knocked out, severe defects in cell morphology result (30), but it is not clear whether a suppressor arose in the $\Delta mtrAB$ mutant. The MtrAB TCSs seem to achieve their regulation of cell surface homeostasis by regulating different sets of genes in different species, again paralleling the YycFG (VicRK) TCSs in low-GC gram-positive bacteria. Last, the MtrAB TCS is always genetically associated with a third component, the LpqB lipoprotein of unknown function (Fig. 1). Similar to the YycHI auxiliary proteins in *B. subtilis*, it has been postulated that LpqB may play roles in signaling to the MtrB histidine kinase (20). Thus, all gram-positive bacteria seem to contain an essential TCS that uses

auxiliary proteins to maintain cell wall and surface homeostasis, possibly in response to changes in cellular signals, stress conditions, or both.

The degrees of essentiality of the YycFG (VicRK) and the MtrAB TCSs in different bacteria bring us back to the issue of whether these TCSs are suitable targets for the development of new antibiotics. In one sense, this question has become somewhat misdirected. Putative YycFG (VicRK)-specific inhibitors that have emerged from some screens (see, e.g., references 25, 35, and 37) would prevent the growth of only low-GC gram-positive, but not high-GC gram-positive or gram-negative, bacteria. In addition, there is a high likelihood of a development of resistance to compounds that inhibit a single target, such as the YycF (VicK) histidine kinase. A far more powerful strategy is to find general inhibitors of histidine kinase autophosphorylation or phosphoryl group transfer between cognate histidine kinases and response regulators. Although this has been a largely unfulfilled challenge (13, 14, 42), there are unusual structural features of these classes of proteins, such as the Bergerat ATP-binding fold in histidine kinases, that make this search attractive (8, 14). That said, having an essential TCS as a target does add to the therapeutic potential of a general TCS inhibitor, and the YycFG (VicRK) and MtrAB TCSs remain attractive targets as part of this larger drug discovery effort. As described above, documented and likely cases of bypass of YycFG (VicRK) TCS function result in defective growth and virulence, which underscores the vital role of this and other TCSs in maintaining cell surface homeostasis in all gram-positive species.

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