A Two-Component Signal Transduction System Essential for
Growth of *Bacillus subtilis*: Implications for
Anti-Infective Therapy†

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A two-component signal transduction system encoded by the yycF and yycG genes is part of an operon containing three genes, *yycH*, *yycI*, and *yycJ*, with no known function and a gene, *yycK*, coding for an HtrA-like protease. This operon was transcribed during growth, and its transcription shut down as the cells approached stationary phase. This decreased transcription was not SpolA dependent. The HtrA protease gene was separately controlled during sporulation from an α² promoter. Studies using insertional inactivation plasmids revealed that neither *yycF* nor *yycG* could be inactivated, whereas the other genes were inactivated without loss of viability. A temperature-sensitive YycF response regulator mutant was isolated and shown to have an H215P mutation in a putative DNA-binding domain which is closely related to the OmpR family of response regulators. At the nonpermissive temperature, cultures of the mutant strain stopped growing within 30 min, and this was followed by a decrease in optical density. Microscopically, many of the cells appeared to retain their structure while being empty of their contents. The essential processes regulated by this two-component system remain unknown. A search of the genome databases revealed YycF, YycG, and YycJ homologues encoded by three linked genes in *Streptococcus pyogenes*. The high level of identity of these proteins (71% for YycF) suggests that this system may play a similar role in gram-positive pathogens.

In order for bacteria to effectively compete and survive, they have to sense environmental conditions and respond accordingly. Two-component systems are a predominant mode of environmental sensing and signal transduction in bacteria. This system of adaptive responses occurs through sensor kinases that mediate reversible phosphorylation events controlling downstream effectors (20, 27). Sensor kinases are generally integral membrane proteins that respond to specific environmental signals, and response regulators are often transcription factors whose affinity for DNA is modulated by phosphorylation. Sensor histidine kinases autophosphorylate on a conserved histidine residue and serve as phosphodonor to an invariant aspartic acid residue on a response regulator protein to which it is paired. The phosphorylated response regulator is thus able to mediate changes in gene expression, leading to the appropriate cellular response.

It is generally believed that two-component systems are used to sense environmental levels of essential substances, such as nitrogen, phosphate, and carbon sources, as well as a variety of nonessential molecules that signal adaptive responses. The discovery of two-component signal transduction involvement in the cell cycle and division of *Caulobacter crescentus* showed for the first time that this mechanism of genetic control had been adopted to regulate vital functions in the cell (7, 22). Because of the therapeutic implications of the discovery, it was important to determine if this adaptation was widespread in bacteria (5). The complete sequence of the *Bacillus subtilis* genome (10) provided a resource to inactivate all of the two-component systems of a single organism by insertional inactivation and determine the phenotype of such mutations. In a study of this kind, a two-component signal transduction system was found that could not be inactivated, and this communication describes the consequences to the cell of inactivation of this system.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmids were constructed as follows. DNA fragments from the *yycFIJK* region were identified by the base pair numbering in the sequence submitted to GenBank under accession no. D78193 (18). For gene disruptions, the PCR was used to amplify regions encoding an internal part of each gene, with *Sac* and *Bam*HI sites added at the ends. After digestion with appropriate enzymes, PCR fragments were ligated to *Sac*I-*Bam*HI-digested pMI03 integrative vectors (21). pJC8 contains a 594-bp internal DNA fragment of the *yycI* gene (bp 35285 to 35879). To obtain pJC10, the chloramphenicol resistance gene from pJC8 was first inactivated by removing the 400-bp *MnlI*- *NcoI* 3'-end fragment; the Klenow-treated plasmid was self-ligated, and then the chloramphenicol resistance gene, without a terminator, on a 955-bp DNA fragment, with no known function and a gene, *yycK*, coding for an HtrA-like protease. This operon was transcribed during growth, and its transcription shut down as the cells approached stationary phase. This decreased transcription was not SpolA dependent. The HtrA protease gene was separately controlled during sporulation from an α² promoter. Studies using insertional inactivation plasmids revealed that neither *yycF* nor *yycG* could be inactivated, whereas the other genes were inactivated without loss of viability. A temperature-sensitive YycF response regulator mutant was isolated and shown to have an H215P mutation in a putative DNA-binding domain which is closely related to the OmpR family of response regulators. At the nonpermissive temperature, cultures of the mutant strain stopped growing within 30 min, and this was followed by a decrease in optical density. Microscopically, many of the cells appeared to retain their structure while being empty of their contents. The essential processes regulated by this two-component system remain unknown. A search of the genome databases revealed YycF, YycG, and YycJ homologues encoded by three linked genes in *Streptococcus pyogenes*. The high level of identity of these proteins (71% for YycF) suggests that this system may play a similar role in gram-positive pathogens.

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to 30417 in D78193) was first obtained from a PCR-amplified fragment (bp 29383 to 30994). Kleow blunted, and cloned into the Smal site of pJM103. The EcoRI-BamHI fragment from this plasmid was then subcloned into EcoRI-BamHI-digested pDH32 and pJM783 to produce the pJC18 and pJC20 vectors, respectively.

To create a conditional mutation, a 474-bp HindIII-BamHI fragment containing the ribosome-binding site and the N-terminal part of yycF gene and place a second copy of the yycF gene is created. The resulting strain was designated JH17040.

**TABLE 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>B. subtilis straina or plasmid</th>
<th>Genotype or descriptionb</th>
<th>Sourcec</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH642</td>
<td>pheA1 trpC2</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>JH646</td>
<td>pheA1 trpC2 spoOA12</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>JH7022</td>
<td>amyE::(yycF-lacZ cat)</td>
<td>pJC16→JH642</td>
</tr>
<tr>
<td>JH7023</td>
<td>yycF::(yycF-lacZ cat)</td>
<td>pJC17→JH642</td>
</tr>
<tr>
<td>JH7024</td>
<td>spoOA12 amyE::(yycF-lacZ cat)</td>
<td>JH70102 DNA→JH646</td>
</tr>
<tr>
<td>JH7025</td>
<td>spoOA12 yycF::(yycF-lacZ cat)</td>
<td>JH70103 DNA→JH646</td>
</tr>
<tr>
<td>JH7026</td>
<td>yycH::pJC11 (Cm')</td>
<td>pJC11→JH642</td>
</tr>
<tr>
<td>JH7027</td>
<td>yycF::pJC12 (Cm')</td>
<td>pJC12→JH642</td>
</tr>
<tr>
<td>JH7028</td>
<td>yycF::pJC13 (Cm')</td>
<td>pJC13→JH642</td>
</tr>
<tr>
<td>JH7029</td>
<td>yycF::pJC14 (Cm')</td>
<td>pJC14→JH642</td>
</tr>
<tr>
<td>JH7030</td>
<td>yycH::(yycF-lacZ cat) Psp ayycFGHIJK ade-16 leu-8 met-5</td>
<td>pJC21→JH642</td>
</tr>
<tr>
<td>Mu8u5u16</td>
<td>ade-16 leu-8 met-5 yycF (H215P) (temperature-sensitive strain)</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>JH7041</td>
<td></td>
<td>5.4-kb PCR→Mu8u5u16</td>
</tr>
</tbody>
</table>

a All JH strains were derived from JH642.
b cat is the chloramphenicol acetyltransferase gene from pC194. Plasmid-encoded antibiotic resistance: Cm', chloramphenicol resistance; Er', erythromycin resistance.
c Arrows indicate transformation.

**RESULTS**

In a survey of the 35 two-component signal transduction systems in B. subtilis (5a), the yycF and yycG genes encoding a two-component system with no known function could not be inactivated, suggesting that this system was essential for growth. Sequence analysis of the yycFG region revealed six open reading frames (18) (Fig. 1). A putative -10 promoter sequence (TATAAT) was identified in the DNA region upstream of the yycF open reading frame, but no sequence substantially similar to the consensus -35 sequence of σ70 promoters was found in this region. Downstream from the yycF and yycG genes, four open reading frames (yycH to yycK) are closely spaced (<22 bp for the three first open reading frames and 69 bp between yycJ and yycK) with no obvious internal transcription terminators. Furthermore, the overlap between open reading frames yycG and yycH (Fig. 1) is consistent with a translational coupling strategy. These data suggested that the six open reading frames may comprise an operon.

**Similarity among yycF, YycG, and two-component proteins.** The deduced sequences of the putative YycG and YycF proteins exhibit structural similarity to histidine kinases and response regulators, respectively, of two-component regulatory systems. The yycG gene encodes a protein of 611 amino acids with a calculated molecular mass of 70 kDa. The C-terminal portion of the protein contains the five blocks of conserved amino acids characteristic of the histidine protein kinase family (Fig. 2); i.e., a conserved histidine, the site of phosphorylation, and the N, G, F, and G boxes, which presumably form a nucleotide-binding surface within the active site (20). The predicted sequence shows the highest similarity to the B. subtilis ResE protein (26) (30% identity) and slightly less similarity to the PhoR protein (25) (28 to 29% identity) (Fig. 2). The hydrophathy profile of the N-terminal putative sensory domain suggests the presence of two stretches of hydrophobic amino acids sufficiently long to span the membrane (data not shown). Amino acids 35 to 182 could be oriented toward the environment in the periplasm, perhaps to sense a specific signal.

The yycF gene encodes a putative protein of 235 amino acids with a calculated molecular mass of 27 kDa. The deduced amino acid sequence shows significant similarity to those of numerous response regulator proteins, such as B. subtilis PhoF (24) (54% identity) and the vancomycin response regulator of Enterococcus faecium (VanR) (3) (43% identity). The YycF response regulator is characterized by a conserved N-terminal domain of approximately 125 amino acids, containing the aspartates and lysine that form the active site.

Response regulators have been classified into subfamilies, according to sequence homologies in their DNA-binding domains (27, 28). On the basis of sequence similarity, the YycF

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**Note:** This transcription is a natural reading of the document, focusing on the key sections and omitting unnecessary details for clarity. The full document includes additional context and in-depth analysis that this summary does not capture. The specific citations and references are not detailed here, as they are not included in the provided text. The table format and figure references are also not included, as they are not part of the natural text. For a complete understanding, the original document should be consulted.
protein should be assigned to the OmpR-PhoB subgroup, to which *B. subtilis* PhoP belongs (11). Proteins from this subclass are thought to bind to promoter sequences that are recognized by the major form of RNA polymerase holoenzyme, corresponding to *B. subtilis* EsaA (27). It thus seems likely that YycF is a DNA-binding transcriptional regulator.

Sequence analysis of the predicted YycH, YycI, YycJ, and YycK products. The predicted products of *yycH*, *yycI*, and *yycJ* do not show any significant matches when compared to the protein sequence in databases. The calculated molecular masses of these putative proteins are 52.5 kDa for YycH, 32.5 kDa for YycI, and 30 kDa for YycJ. Based on their hydrophobicity profiles (data not shown), YycH and YycI could be exported proteins or could be anchored to the membrane by a segment close to the N terminus. YycJ is predicted to be a cytoplasmic protein.

The putative YycK protein displays similarity to members of the HtrA serine protease family (34 to 35% identity with HtrA from *Synechocystis* sp. [9] and *E. coli* [12]). HtrA is involved in the proteolysis of abnormal proteins and is required for resistance to oxidative and heat stress in enteric bacteria (13, 19). YycK contains the motif Gly-Asp-Ser-Gly-Gly-Ala-Lys, which is very similar to the consensus sequence surrounding the active serine residues of the catalytic domains of known serine proteases. The sequence analysis of YycK suggests a 42.8-kDa membrane-anchored protein located outside the cell.

**yycF and yycG are essential genes.** In an effort to determine the functions of YycF and YycG, we used an integrative plasmid to interrupt their genes and analyze the mutant phenotypes. An internal region of each gene was cloned into the pJM103 integrative vector, and the plasmids obtained (pJC8 and pJC9) were used to transform *B. subtilis* JH642. Numerous attempts to integrate these circular plasmids into these genes by a single-crossover event did not yield transformants, suggesting that null mutations in *yycF* or *yycG* may not be constructed.

The same strategy was used for the other open reading frames, *yycH*, *yycI*, *yycJ*, and *yycK*, to determine if mutation of one of them was lethal. By using plasmids pJC11, -12, -13, and -14, transformants were obtained in each case (strains JH17026 to JH17029, respectively), indicating that the deleterious effect observed was due to *yycF* and *yycG* inactivation and not to polar effects on the downstream genes.

In addition, attempts to interrupt *yycF* with a chlorampheni-
predicted transmembrane helix domains are underlined. Conserved motifs present in the histidine kinase family, designated H, N, G1, F, and G2, are indicated by lines above the corresponding sequences.

(pJC16) was linearized and integrated at the nonessential lacZ promoterless locus of B. subtilis, appearing to encode products essential for growth. These data suggest that this two-component system autoregulates its own synthesis, YycF and YycG, which are believed to be involved in the regulation of the expression of the yycK gene products upon entry into stationary phase, the transcriptional fusions were introduced into a spo0A mutant strain (JH466). For construction of strains JH17024 (fusion integrated into the amyE locus) and JH17025 (fusion integrated into the yycF locus), JH466 (spo0A12) was transformed with chromosomal DNAs from JH17022 and JH17023, respectively. The β-galactosidase activities, assayed on cells grown in SM, were similar to those in the wild-type strains (Fig. 3B). Therefore, Spo0A does not appear to play a role in the regulation of the expression of the yycF and yycG genes.

In order to determine if the yycF and yycG gene products autoregulate their own synthesis, β-galactosidase activity was assayed in a strain in which the level of YycF and YycG was controlled by the amount of IPTG added. As seen in Fig. 3C, the β-galactosidase activities, assayed on cells grown in SM, were similar to those in the wild-type strains (Fig. 3B). Therefore, Spo0A does not appear to play a role in the regulation of the expression of the yycF and yycG genes.

FIG. 2. Amino acid sequence alignment of B. subtilis YycG with B. subtilis ResE (SwissProt accession no. P15164) and PhoR (SwissProt accession no. P23545). The predicted transmembrane helix domains are underlined. Conserved motifs present in the histidine kinase family are indicated by lines above the corresponding sequences.
Expression of the \( yycF \) and \( yycK \) promoters. (A) Growth in SM (open symbols) of \( JH17022 \) (○) or \( JH17023 \) (□) and \( \beta \)-galactosidase specific activity of the \( yycF-lacZ \) promoter fusions (solid symbols). (B) Growth in SM (open symbols) of \( JH17023 \) (□), \( JH17024 \) (\( spo0A \) strain with the \( yycF-lacZ \) fusion integrated into the \( amyE \) locus) (●), \( JH17025 \) (\( spo0A \) mutant with the fusion in the chromosomal \( yycF \) locus) (◇), and \( \beta \)-galactosidase specific activity of the promoter fusion (solid symbols). (C) Effect of variations in the level of the \( YycF \) and \( YycG \) proteins on the growth in SM (open symbols) and expression of the operon (solid symbols) of \( JH17040 \) without IPTG (○), with 50 \( \mu \)M IPTG (△), with 0.5 mM IPTG (X), and with 1 mM IPTG (+). Growth of \( JH17025 \) (◇) is plotted as a control. (D) Growth in SM (open symbols) and \( \beta \)-galactosidase specific activity (solid symbols) of \( JH642 \) with the \( yycK-lacZ \) fusion integrated into the \( amyE \) locus (pJC18) (□) or into the \( yycK \) locus (pJC20) (○).

**FIG. 3.** Expression of the \( yycFG \) and \( yycK \) promoters. (A) Growth in SM (open symbols) of \( JH17022 \) (○) or \( JH17023 \) (□) and \( \beta \)-galactosidase specific activity of the \( yycF-lacZ \) promoter fusions (solid symbols). (B) Growth in SM (open symbols) of \( JH17023 \) (□), \( JH17024 \) (\( spo0A \) strain with the \( yycF-lacZ \) fusion integrated into the \( amyE \) locus) (●), \( JH17025 \) (\( spo0A \) mutant with the fusion in the chromosomal \( yycF \) locus) (◇), and \( \beta \)-galactosidase specific activity of the promoter fusion (solid symbols). (C) Effect of variations in the level of the \( YycF \) and \( YycG \) proteins on the growth in SM (open symbols) and expression of the operon (solid symbols) of \( JH17040 \) without IPTG (○), with 50 \( \mu \)M IPTG (△), with 0.5 mM IPTG (X), and with 1 mM IPTG (+). Growth of \( JH17025 \) (◇) is plotted as a control. (D) Growth in SM (open symbols) and \( \beta \)-galactosidase specific activity (solid symbols) of \( JH642 \) with the \( yycK-lacZ \) fusion integrated into the \( amyE \) locus (pJC18) (□) or into the \( yycK \) locus (pJC20) (○).
ment was used to transform strain Mu8u5u16 to Ade. About 3,200 transformants were transferred to replicate plates and incubated at 30°C or 47°C. One strain did not grow at 47°C on either minimal medium or SM plates.

In order to locate the mutation giving the phenotype, chromosomal DNA was prepared from the thermosensitive mutant strain (JH17041) and used as template DNA in PCRs to amplify the entire yycF gene (the fragment in pJC15 [Fig. 1]) and the entire yycG gene (see Materials and Methods). The amplified DNA fragments were then cloned and sequenced. Only one alteration was found, and that was localized in the yycF gene. Mutation of A to C at position 35303 (sequence with GenBank accession no. D78193) changed histidine 215 to a proline.

To determine if this mutation was responsible for the thermosensitive phenotype, the PCR-amplified fragment containing the yycF mutant gene was cloned into the integrative pJM103 vector and used to transform JH642. The fragment was identical to that cloned into pJC15 (Fig. 1). Among the transformants, 35% displayed a thermosensitive phenotype. The same experiment, when done with the identical PCR-amplified fragment from the parental strain, did not result in thermosensitivity.

A second experiment was done to confirm that the yycF mutation was responsible for the thermosensitive phenotype. The fragment in plasmid pJC19 (Fig. 1) was obtained in two pieces by two PCRs from the DNA of thermosensitive strain JH17041, and they were ligated to the chloramphenicol resistance gene and cloned into pJM105A. This placed the Cmr cassette upstream of the promoter and allowed the insertion of the entire fragment into the chromosome as a double-cross-over event. Sequence analyses confirmed the presence of only a single mutation in yycF, as previously described. Transformation of this plasmid in JH642 gave 30% thermosensitive transformants.

Growth and phenotypic characteristics of the thermosensitive strain. The thermosensitive and parental strains were grown in LB broth at 30°C until an optical density at 525 nm (OD525) of 0.4 was reached; when half of each culture was transferred to 47°C. At 30°C, both strains grew similarly (Fig. 4). Upon a shift to 47°C, the thermosensitive strain stopped growing after approximately 30 min and the OD525 decreased. When cultures were observed with a microscope, the thermosensitive strain at the permissive temperature produced chains of cells characteristic of early exponential-phase cultures that persisted longer than parental strains under the same conditions (Fig. 5A). Within an hour from when the cultures were shifted to the nonpermissive temperature, the chains of the thermosensitive mutant cells were interspersed with empty sections, as if some of the cells had lost their cytoplasmic contents and only the cell wall remained (Fig. 5B). The parental strain showed none of this behavior and had separated into single or double cells after 1 h at 47°C (data not shown).
DISCUSSION

On the basis of the sequence similarity of its C-terminal domain, the YycF response regulator has been assigned to the OmpR subfamily. The crystal structure of the E. coli OmpR C-terminal domain revealed that members of the subfamily belong to the winged helix-turn-helix family of DNA-binding proteins (Fig. 6) (15). Of the 17 residues known to make up the hydrophobic core of OmpR, all are either identical or conserved hydrophobic in YycF. Analogy with other winged helix-turn-helix proteins and correlation with mutagenesis data have allowed the identification of important structural features: a recognition helix, a wing involved in DNA binding, and an extensive loop preceding the recognition helix involved in interaction with the α subunit of RNA polymerase (15). The residues thought to be important for DNA binding in OmpR, such as Arg182, Val203, and Thr224, are identical in YycF, and Ser200 is replaced with a similar amino acid, threonine. The thermosensitive mutation of YycF was localized to the loop connecting the α3 helix to the β6 and β7 C-terminal strands. Substitution of a proline for a histidine at this site may perturb the DNA-binding properties of this region by destabilizing the interaction of β6 and β7 with the rest of the molecule at elevated temperature.

The YycG kinase is related to the ResE and PhoR kinases of B. subtilis. All of these kinases appear to have two membrane-spanning regions which form a putative periplasmic loop of about 140 amino acids. The active-site histidine of the cytoplasmic catalytic domain is preceded by about 160 amino acids forming a domain with no known function in all cases. The highest homology among these proteins is found in the catalytic domain, with less in the cytoplasmic domain. Very little amino acid sequence conservation was found among all three kinases in the putative periplasmic domain, but YycF and ResE have 34% identical and similar residues in this region. If all of these proteins evolved from an ancestral protein, there appears to be more pressure to conserve the histidine- and ATP-binding regions.

The YycF response regulator is likely to regulate several genes which remain unidentified. Clearly, loss of this activity in the thermosensitive mutant results in rapid cessation of growth. The cellular effect of this loss as revealed by microscopy was the generation of empty cells which maintained their structural rigidity. This was especially apparent in septated chains of cells characteristic of low-density cultures when transferred to the nonpermissive temperature. The molecular basis of this phenomenon is unknown. YycF may control some genes whose products are essential for normal cell growth and

FIG. 6. Sequence alignment of the DNA-binding domains of B. subtilis YycF and E. coli OmpR (SwissProt accession no. P03025). A schematic diagram of the secondary structure of OmpR is shown (15). The dots mark residues corresponding to the OmpR hydrophobic core. The position of thermosensitive mutation is shaded.

FIG. 7. Organization of the yycFG-like genes in S. pyogenes M1 GAS (sequenced at Oklahoma University [http://www.genome.ou.edu/strep.html]) and amino acid sequence alignment of the predicted products (YycFGJSt) with the homologs in B. subtilis (YycFGJbs). Conserved motifs in the histidine kinase proteins are indicated by lines above the corresponding sequences.
are likely to be integrated with other cell cycle-dependent events, since transcription of the operon containing the yycF and yycG genes occurs only during growth and is shut off as the cells approach stationary phase. This shutdown is not mediated by the sporulation regulator Spo0A but may be due to loss of an inducing signal from, or dependent on, cell growth. Clearly, there are many unknown features of this system, including what signals regulate the YycG kinase.

Essential two-component signal transduction systems have been found in C. crescentus. In C. crescentus, a system of histidine kinases and response regulators controls cell division and motility (7, 22). The growth signals that this system interprets to affect cell division remain obscure. Similarly, YycF and YycG are likely to respond to growth signals in B. subtilis. In fact, very little is known about how cells coordinate processes such as cell wall and membrane growth with DNA replication.

yycF proteins suggest that they carry out the same function in the two organisms, since transcription of the operon containing the yycF and yycG genes should effectively curtail growth and result in rapid cell death. This system may be a target for a series of two-component signal transduction inhibitors with bactericidal properties that have recently been described. These inhibitors were especially effective on gram-positive pathogens, such as methicillin-resistant Staphylococcus aureus. Since the genome sequence of S. aureus is proprietary, it cannot be determined by us whether a YycF equivalent exists in this organism, but the S. pneumoniae results suggest that such a system is common to gram-positive microorganisms (4). Resistance to these inhibitors was multifactorial, indicating that more than one target was being affected by the inhibitors (4). This may indicate that more than one kinase is essential for growth or that general kinase inhibition is a lethal event. The ResE-ResD signal transduction system to which YycG and YycF are closely related is essential for growth in the absence of glucose. How many other systems of their type, when mutated singly or in combination, would have this phenotype has yet to be determined. The presence of similar two-component systems in gram-positive pathogens suggests that kinase or response regulator inhibitors would be effective bactericidal anti-infective agents (4, 5, 14).

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