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Identification of Genes Controlled by the Essential YycG/YycF Two-Component System of *Staphylococcus aureus*

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The YycG/YycF essential two-component system (TCS), originally identified in *Bacillus subtilis*, is very highly conserved and appears to be specific to low-G+C gram-positive bacteria, including several pathogens such as *Staphylococcus aureus*. By studying growth of *S. aureus* cells where the *yyc* operon is controlled by an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, we have shown that this system is essential in *S. aureus* during growth at 37°C and that starvation for the YycG/YycF regulatory system leads to cell death. During a previous study of the YycG/YycF TCS of *B. subtilis*, we defined a potential YycF consensus recognition sequence, consisting of two hexanucleotide direct repeats, separated by five nucleotides [5′-TGT(A/T)A(A/T/C)-N_5-TGT(A/T)A(A/T/C)-3′]. A detailed DNA motif analysis of the *S. aureus* genome indicates that there are potentially 12 genes preceded by this sequence, 5 of which are involved in virulence. An in vitro approach was undertaken to determine which of these genes are controlled by YycF. The YycG and YycF proteins of *S. aureus* were overproduced in *Escherichia coli* and purified. Autophosphorylation of the YycG kinase and phosphotransfer to YycF were shown in vitro. Gel mobility shift and DNase I footprinting assays were used to show direct binding in vitro of purified YycF to the promoter region of the *ssaA* gene, encoding a major antigen and previously suggested to be controlled by YycF. YycF was also shown to bind specifically to the promoter regions of two genes, encoding the IsaA antigen and the LytM peptidoglycan hydrolase, in agreement with the proposed role of this system in controlling virulence and cell wall metabolism.

The major human pathogen *Staphylococcus aureus* is responsible for a broad spectrum of infections, ranging from food poisoning and superficial skin abscesses to more serious diseases such as pneumonia, meningitis, endocarditis, septicemia, and toxic shock syndrome. The unique adaptive potential displayed by *S. aureus* to environmental conditions and stress has made it one of the main causes of nosocomial infections, emphasized by the rapid emergence of multiple-antibiotic-resistant strains over the past few decades. Until now, vancomycin had remained the weapon of last resort, but the recent appearance of the *vanA* vancomycin resistance gene cluster in *S. aureus* highlights the growing threat this bacterium poses to human health and the urgent need to develop novel therapeutic approaches (5). In many cases, virulence gene expression in pathogens is controlled by two-component histidine kinase-response regulator signal transduction systems (TCSs) (9), yet apart from the well-studied AgrC/AgrA system of *S. aureus* (30) and the CovS/CovR system of *S. pyogenes* (16), little is known about the function of TCSs in gram-positive pathogens. The high degree of conservation among TCSs, their ubiquitous nature, and the fact that several are essential for cell viability have made them an attractive target for novel classes of antimicrobial compounds (3, 37). The YycG/YycF TCS is specific to low-G+C gram-positive bacteria, where it is the most highly conserved TCS. It has been shown to be essential in *Bacillus subtilis* and *Streptococcus pneumoniae*, and a thermosensitive mutation of the system in *S. aureus* has been shown to be lethal at nonpermissive temperatures (11, 23, 27, 40, 42). Attempts to inactivate the *yycFG* genes in *Streptococcus pyogenes* and *Listeria monocytogenes* have failed, suggesting that the system is also essential in these bacteria (12, 19).

The *yycG/yycF* genes are expressed during exponential growth and rapidly shut off at entry into stationary phase, suggesting that this system is active, and also necessary, during the exponential growth phase (11). All of the YycG kinase orthologs are similar in domain organization and contain a PAS domain, as determined by using the SMART database (36). PAS domains are signaling modules essentially found in proteins involved in signal transduction. They have been involved in monitoring changes in light, redox potential, oxygen, small-ligand concentration, and more generally, intracellular energy status (39).

Previous studies on the YycG/YycF system have shown that it is involved in competence development and virulence in *S. pneumoniae* (10, 42). In *S. aureus*, the YycG/YycF system is thought to play a role in membrane permeability, and a temperature-sensitive mutant of *S. aureus yycF* exhibited attenuated virulence in an in vivo model (27). Furthermore, links were established among YycG/YycF, resistance to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics, and a secreted antigen, SsaA (26). The YycG/YycF ortholog system in *B. subtilis* is composed of the YycG histidine kinase and the YycF response regulator (11). Although the exact role of the YycG/YycF system in *B. subtilis* remains to be established, it has been suggested to be involved in cell division by modulating the expression of the *ftsAZ* operon (13). During a previous study of the YycG/YycF TCS of *B. subtilis*, we defined a potential YycF consensus recognition sequence consisting of two hexanucleotide direct repeats separated by five nucleotides [5′-TGT(A/T)A(A/T/C)-N_5-TGT(A/T)A(A/T/C)-3′] (18). In

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TABLE 1. Oligonucleotides used in this study

Name	Sequence
OSA34	5′-TCTAGATATTAATGATTTAAGAAAAGAGG-3′
OSA35bis	5′-AGATCTCCAGTGTCTTGTGCTGGTTGTGAG-3′
OSA54	5′-TTTTCTGATTAAAAGTCCTATATTATGCAC-3′
OSA55	5′-ATCCTGCAGTTGCGATAGTAGCTGTAGCGA-3′
OSA90	5′-GAGAAGCATATCACTATTAGAAAGTTG-3′
OSA91	5′-GCGCCATTGTAAATGTAGCGAAGCCC-3′
OSA92	5′-GGGTTGATTGGATTCTAAAGGGCACATATTTC-3′
OSA93	5′-GCGTAACCTGTTACACCTAATGCCACTGC-3′
TM314	5′-CCACCATGGCTAGAAAAGTTGTTGTAGTTG-3′
TM315	5′-CTCCTCGAGCTCATGTTGTTGGAGGAAATATCCA-3′
TM316	5′-GGTGGTCTCCCATGTCCAGAGGTAACTATACGC-3′
TM317	5′-CTCCTCGAGTTCATCCCAATCACCGTCTTCAATG-3′

addition to the *ftsAZ* cell division genes, many of the genes controlled by the YycG/YycF system in *B. subtilis* play a role in cell wall metabolism (*tagA*/*tagD* in teichoic acid biosynthesis; *yocH*, encoding a potential autolysin; and *ykvT*, encoding a potential spore cortex lytic enzyme) (18).

Since the YycF response regulators of *B. subtilis* and *S. aureus* are highly conserved (74% amino acid sequence identity), particularly in the recognition helix of the OmpR-type winged helix-turn-helix DNA-binding domains, it is highly likely that the two regulators recognize the same DNA sequence. With our previously defined consensus sequence, a detailed DNA motif analysis of the *S. aureus* genome was performed and a list of genes potentially regulated by the YycG/YycF system was established. These encode proteins involved in cell wall metabolism, membrane-bound transport systems, and pathogenicity, including two major antigenic proteins, SsaA and IsaA, and three proteins implicated in interactions between the bacteria and the host matrix. We have shown that YycF of *S. aureus* binds directly to the regulatory upstream regions of three of these genes, indicating that the regulator recognizes the same consensus established in *B. subtilis*. Furthermore, these results suggest that the YycG/YycF system regulates the expression of several virulence genes by direct interaction with their promoter regions. This is the first report of genes directly controlled by the YycG/YycF system of *S. aureus*, whose essential nature in a major pathogen makes it a prime target for the development of novel antibacterial compounds.

MATERIALS AND METHODS

Bacterial strains and growth media. *Escherichia coli* K-12 strain TG1 (14) was used for cloning experiments, and *E. coli* strain BL21λDE3 (38) was used for protein overexpression and purification. *E. coli* strains were grown in Luria-Bertani medium and transformed by electroporation (35). *S. aureus* RN4220 (21) and its derivatives were grown in Trypticase soy broth (TSB) medium and transformed by electroporation, with selection on plates supplemented with chloramphenicol (10 $\mu g \cdot ml^{-1}$).

Plasmids and plasmid constructions. Standard procedures were used for DNA manipulation (35). Plasmid pDH88 was used for isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible gene expression (17). Plasmid pET28/16 (6), a derivative of plasmid pET28a (Novagen), was used for protein overexpression and purification.

To generate a strain in which the *yyc* operon was placed under the control of the inducible P*spac* promoter, a 432-bp fragment generated by PCR with primers OSA34 and OSA35bis (Table 1), carrying the ribosome-binding site and 5′ portion of *yycF*, was digested with *Xba*I and *Bgl*II and cloned into pDH88 to generate pSD3-3. The circular plasmid was then transformed into *S. aureus* strain RN4220, and integration of the plasmid through a single crossover event generated strain RN4220P*spac-yycF*, in which the *yycF* operon was placed under P*spac* control.

The YycG histidine kinase has two amino-terminal transmembrane domains, located between residues 14 and 34 and between residues 183 and 203, as determined with von Heijne's algorithm (7). YycF and ′YycG (isolated cytoplasmic histidine kinase domain, residues 220 to 609) were overproduced with plasmids pETyycF and pETyycG, respectively, constructed by cloning a PCR-generated *Nco*I/*Xho*I DNA fragment corresponding to the *yycF* coding sequence (715 bp, PCR TM314/TM315) or a PCR-generated *Bsa*I/*Xho*I DNA fragment corresponding to the *′yycG* coding sequence (1,189 bp, PCR TM316/TM317) between the *Nco*I and *Xho*I sites of plasmid pET28/16, replacing the stop codons with an *Xho*I restriction site. The *Bsa*I enzyme cleaves after its restriction site, generating a DNA fragment whose cohesive end was defined to be compatible with *Nco*I. This allows the creation of translational fusions adding six histidine residues to the carboxy terminus of the corresponding protein, placing expression of the genes under the control of a T7 bacteriophage promoter.

Overproduction and purification of ′YycG and YycF. Plasmids pETyycF and pETyycG were introduced into a BL21/λDE3 strain, in which the T7 RNA polymerase gene is under the control of the inducible *lacUV5* promoter, which also carries the pREP4 plasmid, allowing coproduction of the GroESL chaperonin, in order to optimize recombinant protein solubility (2). The resulting strains were grown in 2 liters of Luria-Bertani medium at room temperature, expression was induced during the mid-exponential growth phase by addition of 1 mM IPTG, and incubation was pursued for 4 h. Cells were centrifuged at 10,800 × *g* for 30 min and resuspended in 1/50 of the culture volume of buffer I (50 mM NaH_2PO_4 [pH 8], 300 mM NaCl, 20 mM imidazole). Cells were disrupted by sonication, and cell debris was removed by two consecutive 30-min centrifugation steps at 17,200 × *g*. *E. coli* crude protein extracts were loaded onto a 0.2-ml Ni-nitrilotriacetic acid agarose (Qiagen) column equilibrated with buffer I. The column was washed with 10 volumes of buffer II (50 mM NaH_2PO_4 [pH 6], 300 mM NaCl, 30 mM imidazole), and the proteins were eluted with an imidazole gradient (30 to 500 mM). Fractions were pooled and dialyzed against buffer III (50 mM NaH_2PO_4 [pH 8], 300 mM NaCl, 50% glycerol) to remove imidazole and concentrate the protein solution approximately fourfold.

Protein phosphorylation assays. For ′YycG autophosphorylation, 1.5 μg of protein was incubated for 1 min at room temperature in 10 μl of phosphorylation buffer (100 mM Tris HCl [pH 8], 200 mM KCl, 4 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 3.5% glycerol, 2.5 μM ATP, 4 μCi of [γ-^{32}P]ATP). For phosphotransfer from ′YycG to YycF, equal amounts of both proteins were incubated together and the reaction was initiated by the addition of a radiolabeled ATP mixture as described above. Reactions were stopped by adding 2 μl of sodium dodecyl sulfate (SDS) loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 12% acrylamide gels, followed by autoradiography.

Gel shift assay. A 381-bp DNA fragment corresponding to the *ssaA* promoter region was generated by PCR with *Pwo* polymerase (Roche) and oligonucleotides OSA54 and OSA55 (Table 1). Labeling, DNA binding, and electrophoresis were performed as described previously (8).

DNase I footprinting. DNA fragments corresponding to the promoter regions of *ssaA* (381 bp, OSA54/OSA55), *isaA* (311 bp, OSA92/OSA93), and *lytM* (430 bp, OSA90/OSA91) were generated by PCR with *Pwo* polymerase (Roche) and the indicated oligonucleotide pairs (for sequences, see Table 1). Labeling of DNA fragments was performed as described previously (8). YycF binding to DNA (5×10^4 cpm per reaction mixture) was performed at room temperature in a buffer containing 25 mM NaH_2PO_4 (pH 8), 50 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol. DNase I treatment and electrophoresis were then performed as described previously (8).

Database comparisons and sequence analysis. Computations were performed with the TopPredII (7) program and the SubtiList and AureoList relational databases (http://genolist.pasteur.fr) (29). Sequence comparisons with the GenBank database were accomplished with the National Center for Biotechnology Information BLAST2 (1) web server by using the default parameter values provided.

RESULTS

Growth and stress resistance of a conditional *yycFG* mutant. Growth of a conditional P*spac-yycF* mutant was monitored in TSB medium. An overnight culture of the mutant was inoculated with or without IPTG at an optical density at 600 nm (OD_{600}) equal to 0.005 and incubated at 37°C. After six generations without IPTG, growth was arrested, indicating that the system is essential at physiological temperatures (Fig. 1A). Interestingly, even after prolonged incubation in the absence

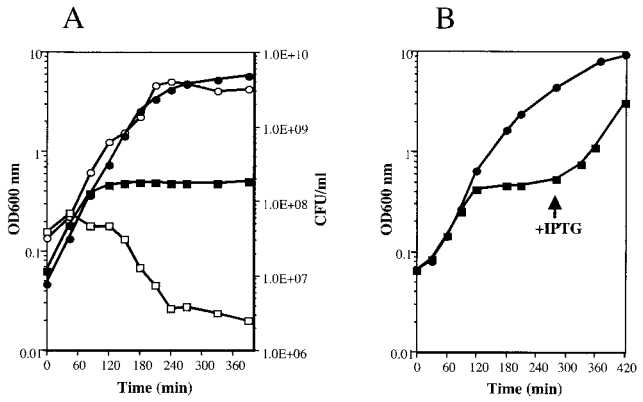

FIG. 1. IPTG-dependent growth of the conditional RN4220P*spac-yycF* mutant strain. An overnight culture in TSB plus 1 mM IPTG was diluted to an OD_{600} of 0.005 in TSB medium with (circles) or without (squares) 1 mM IPTG. (A) OD_{600} values (solid symbols) and numbers of CFU per milliliter (open symbols) were measured in TSB medium. For measurement of the number of CFU per milliliter, cells were appropriately diluted in TSB liquid medium and plated on TSB plates with 1 mM IPTG. (B) OD_{600} was measured with (circles) or without (squares) 1 mM IPTG. At the indicated times, 1 mM IPTG was added to the culture without IPTG. Each experiment was repeated three times, and the values obtained did not vary significantly.

of the inducer, the cells did not undergo lysis (Fig. 1A). The number of CFU was monitored in the same assay by plating the cultures on TSB medium plus IPTG. Although no cell lysis was observed in the absence of IPTG, the number of bacteria able to form colonies was drastically decreased, leading to 95% cell death 3 h after cessation of growth, suggesting that absence of *yycFG* expression is lethal for the cell (Fig. 1A).

Microscopic examination of the mutant cells cultivated in the absence of IPTG did not reveal any anomalous cell morphology as previously observed for a conditional mutant of *B. subtilis* (data not shown) (11, 13). In order to test the capacity of the surviving viable cells to resume a normal growth rate, IPTG was reintroduced 160 min after the growth arrest. After a lag phase, the surviving cells resumed exponential growth, indicating that, as on plates, the living cells recover the ability to divide once the YycG/YycF proteins are produced (Fig. 1B).

The *S. aureus* RN4220P*spac-yycF* strain has a normal growth rate in TSB medium containing 1 mM IPTG, with a doubling time of 30 min. In order to identify stress conditions that require a high level of YycG/YycF in the cell, we determined the minimal inducer concentration that allows normal growth under laboratory culture conditions (rich medium at 37°C with aeration). We determined that the cells have the same doubling time when grown with 1 mM or 50 μM IPTG (data not shown).

We then compared the resistance of cells grown with 1 mM or 50 μM IPTG when exposed to alcohol stress (6% ethanol), salt stress (5% NaCl), high temperature (45°C), and acidic or basic stress (pH 4 or pH 10, respectively). Under these conditions, the cells grew equally well with minimal or maximal inducer concentrations, suggesting that the YycG/YycF system does not play an essential role in resistance to these stresses (data not shown).

Genome-based prediction of the YycG/YycF regulon. The YycF regulator of *S. aureus* is very similar to its ortholog in *B. subtilis*. They have 75% amino acid sequence identity, and the winged helix-turn-helix DNA-binding domains have 30 identical amino acids out of 31 (Fig. 2). This very high level of identity between the two regulators suggested that they may recognize the same DNA sequences. We previously identified a consensus YycF recognition sequence in *B. subtilis*, consisting of two hexanucleotide direct repeats separated by five nucleotides [5′-TGT(A/T)A(A/T/C)-N_5-TGT(A/T)A(A/T/C)-3′] (18). We performed an in silico search for this motif in the intergenic regions of the complete *S. aureus* N315 genome with the Search Pattern function of the AureoList database (http://genolist.pasteur.fr/AureoList/) and have established a list of genes potentially regulated by YycG/YycF. Once matches located between convergent genes were eliminated, 34 potential binding sites on either strand within the 400-bp region upstream from the translation initiation codon were identified, corresponding to 31 genetic loci. Among these, only 12 genes encode putative proteins whose potential function can be deduced from sequence similarities (Table 2), whereas the remaining 19 are of unknown function (SA0100, SA0129, SA0364, SA0502, SA0570, SA0618, SA0620/SA0621, SA0674, SA0682, SA0710, SA0913, SA1898, SA1945, SA2097, SA2118, SA2305, SA2353, SA2439, and SA2481). Of the 12 genes potentially regulated by the YycG/YycF system listed in Table 2, 5 have been described as pathogenicity factors: 2 encode major antigens, SsaA and IsaA, and 3 others encode bacterial proteins implicated in interactions with the host matrix (a sialoprotein-binding protein, an elastin-binding protein, and a staphylokinase precursor). One gene, *lytM*, encodes a peptidoglycan hydrolase, a function shown to be regulated by the YycG/YycF system in *B. subtilis* (18, 33). We note that 3 of the 19 genes of unknown function preceded by a potential YycF binding site encode proteins with significant similarities to the SsaA antigen (SA0620, SA2097, and SA2353).

Purification and phosphorylation of ′YycG and YycF. In order to determine whether genes on this list are directly regulated by YycF, the ′YycG and YycF proteins were overproduced and purified. The *yycF* coding sequence was cloned into plasmid pET28/16, creating a translational fusion adding six histidine residues to the carboxy terminus of YycF and placing the gene under the control of an inducible T7 bacteriophage promoter (see Materials and Methods). In order to overproduce ′YycG, a DNA fragment encoding the isolated

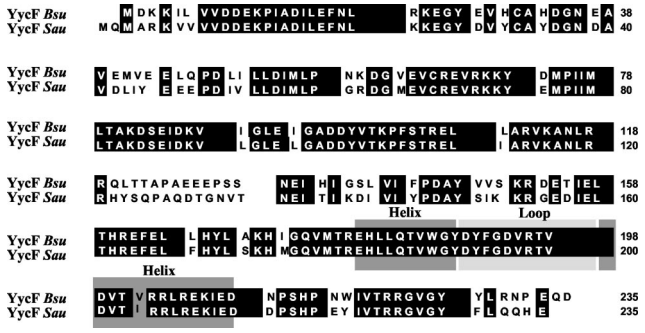

FIG. 2. Amino acid sequence alignment of the *B. subtilis* and *S. aureus* YycF proteins. Conserved residues are shaded, and the DNA-binding winged helix-turn-helix domains are boxed.

TABLE 2. Alignment of nucleotide sequences of putative YycF-regulated promoter regions

Gene	Sequence[a]	Function
*lytM**(SA0265)	**TGTAAT**GACAA**TGTAAT**...N_{158}...ATG	Peptidoglycan hydrolase
prs (SA0458)	**TGTAAT**ATTAA**TGTAAT**...N_{103}...ATG	Ribose-phosphate pyrophosphokinase
sdrD (SA0520)	**TGTTAC**AGAAA**TGTAAT**...N_{267}...ATG	Sialoprotein-binding protein
hu (SA1305)	**TGTAAT**GCTTG**TGTTAA**...N_{74} ...ATG	DNA-binding protein II
ebpS (SA1312)	**TGTAAA**ATCAT**TGTAAT**...N_{38} ...ATG	Elastin-binding protein
phoP (SA1516)	**TGTAAA**AAATG**TGTAAA**...N_{93} ...ATG	Transcriptional regulatory protein
sak (SA1758)	**TGTTAA**ATATT**TGTTAA**...N_{176}...ATG	Staphylokinase precursor
*isaA** (SA2356)	**TGTAAA**GAAAG**TGTAAT**...N_{156}...ATG	Immunodominant antigen A
opp-2B (SA1214)	**GTTACA**CTGCA**GTAACA**...N_{265}...ATG	Oligopeptide transporter membrane permease domain
ndhF (SA0411)	**TTTACA**AAATC**TTTACA**...N_{369}...ATG	NADH dehydrogenase
SA1221	**TTTACA**TTTTC**TTAACA**...N_{129}...ATG	Thioredoxin reductase
*ssaA** (SA2093)	**ATTACA**AAACG**TTAACA**...N_{136}...ATG	Staphylococcal secretory antigen
	ATTACAAATTT**GTAACA**...N_{265}...ATG	

[a] The direct repeats of the potential YycF binding sites on either strand are shown in bold and indicated by arrows. Genes where direct binding by YycF was demonstrated are indicated by asterisks. The *ssa* gene promoter region contains two binding sites for YycF.

cytoplasmic histidine kinase domain was cloned into pET28/16, adding six histidine residues to the carboxy terminus of the protein (see Materials and Methods). His-tagged YycF and ′YycG were purified in a single step with an Ni-nitrilotriacetic acid agarose column (see Materials and Methods), and SDS-PAGE analysis revealed a purity greater than 90% (Fig. 3A, lanes 4 and 6, respectively). The purified ′YycG protein displayed the expected apparent molecular mass (approximately 44.8 kDa) (Fig. 3A, lane 6), whereas the recombinant YycF protein migrated slightly higher than the expected size (approximately 30 kDa instead of the deduced molecular mass of 28.2 kDa) (Fig. 3A, lane 4). Such anomalous electrophoretic migration has previously been reported for its ortholog in *B. subtilis* (18).

Autophosphorylation of the ′YycG histidine kinase was demonstrated by incubating the protein with [γ-^{32}P]ATP and then subjecting it to SDS-PAGE and autoradiography, as shown in Fig. 3B, lane 1. When the purified YycF and ′YycG proteins were incubated together, autophosphorylation of ′YycG and phosphotransfer to YycF were observed upon addition of [γ-^{32}P]ATP, followed by SDS-PAGE and autoradiography (Fig. 3B, lanes 2 to 7). Phosphorylation of YycF by ′YycG reached equilibrium in approximately 10 min (Fig. 3B, lane 5).

YycF binds specifically to the *ssaA* promoter region. As mentioned above, *ssaA* appears among the genes listed as potential members of the YycG/YycF regulon. In a previous report, it has been shown that the hypersensitivity to MLS_B antibiotics of a temperature-sensitive *yycF* mutant could be suppressed by *ssaA* overexpression, suggesting that *ssaA* expression could be regulated by YycF (26). To test this hypothesis, we have carried out assays of YycF binding to the *ssaA* promoter region. Gel mobility shift assays were performed with the purified YycF protein and a 381-bp radiolabeled DNA fragment corresponding to the *ssaA* promoter region and synthesized by PCR with oligonucleotides OSA54 and OSA55 (Table 1). The appearance of two different-sized protein-DNA complexes suggests that two YycF molecules can bind to the *ssaA* promoter region (Fig. 4A). DNase I footprinting assays

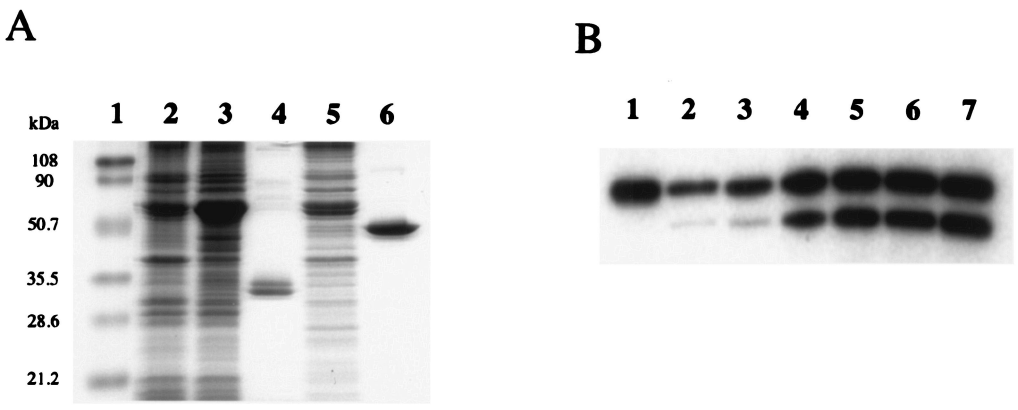

FIG. 3. Purification and phosphorylation of ′YycG and YycF. (A) SDS-PAGE analysis of crude extracts from *E. coli* BL21/λDE3 carrying pET28/16 (lane 2), pETyycF (lane 3), or pETyycG (lane 5). Purified proteins (approximately 50 pmol) were loaded in lane 4 (YycF) and lane 6 (′YycG). Molecular size standards were loaded in lane 1. (B) In vitro phosphorylation of ′YycG and YycF. Lane 1, ′YycG incubated with [γ-^{32}P]ATP for 1 min at room temperature. ′YycG and YycF were incubated for various times following the addition of [γ-^{32}P]ATP. Lanes 2 to 7, 0.5, 1, 5, 10, 15, and 20 min, respectively.

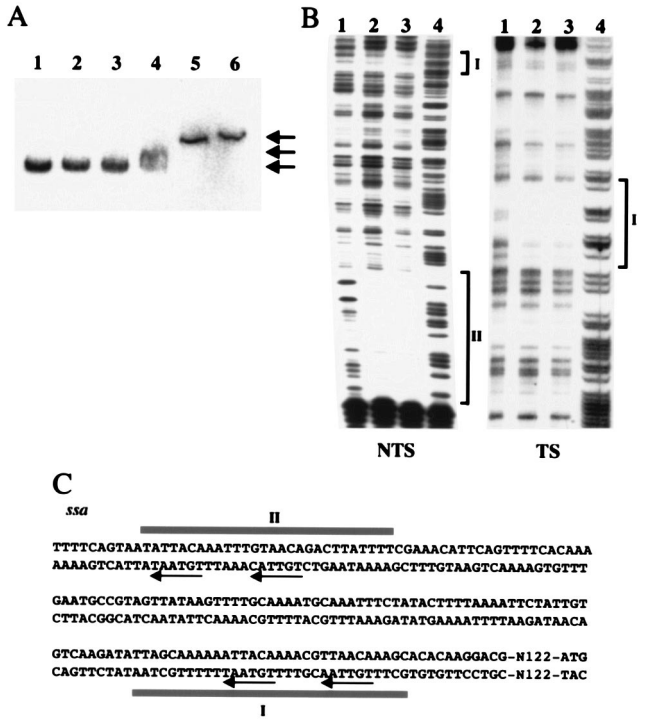

FIG. 4. Gel mobility shift assay and DNase I footprinting analysis of YycF binding to the *ssaA* promoter region. (A) DNA-binding reactions were performed with 0.1 pmol of a radiolabeled DNA fragment corresponding to the *ssaA* promoter region (−340 to +40 with respect to the translation initiation site) and purified YycF. Lanes: 1, no protein; 2, 26 pmol; 3, 34 pmol; 4, 53 pmol; 5, 68 pmol; 6, 106 pmol. (B) DNase I footprinting of YycF bound to the *ssaA* promoter region. Lanes contain approximately 0.5 pmol (5×10^4 cpm per reaction mixture) of labeled nontemplate or template strand (NTS and TS, respectively) of *ssaA* (−340 to +40). Lanes: 1, no protein; 2, 140 pmol; 3, 212 pmol; 4, A+G Maxam and Gilbert reaction. Brackets indicate proximal and distal regions protected by YycF from DNase I cleavage (I and II, respectively). (C) Nucleotide sequences of the *ssaA* promoter region. Regions protected by YycF from DNase I cleavage are shown by bars, and the conserved repeats are indicated by arrows.

were performed to precisely determine the location of the YycF binding site(s). As shown in Fig. 4B, YycF binds two distinct regions, extending from position −283 to position −255 and from −164 to −133 relative to the translation start site (Fig. 4C), in agreement with the two different protein-DNA complexes observed in the gel mobility shift experiments. These two regions each contain the direct repeat predicted to be a YycF target site (Table 2). These data constitute the first demonstration of direct binding of the YycF regulator on a promoter region in *S. aureus*. Furthermore, they provide a direct link between *ssaA* expression and the YycG/YycF system, since it seems that *ssaA* transcription is directly regulated by YycF.

Binding of YycF in two additional promoter regions predicted to contain YycF binding sites. In addition to *ssaA*, four genes closely linked to *S. aureus* virulence have the YycF consensus sequence in their promoter regions: *sdrD*, encoding a sialoprotein-binding protein; *ebpS*, encoding an elastin-binding protein; *sak*, encoding a staphylokinase precursor; and *isaA*, encoding immunodominant antigen A (Table 2). Furthermore, as described in *B. subtilis* (18), it seems that the YycG/YycF system also regulates cell wall metabolism in *S. aureus* via regulation of *lytM*, which encodes a peptidoglycan hydrolase (33). DNase I footprinting assays were performed on radiolabeled DNA fragments corresponding to the *lytM* and *isaA* promoter regions, generated by PCR with oligonucleotides OSA92/OSA93 (*isaA*) and OSA90/OSA91 (*lytM*) (the oligonucleotide sequences are listed in Table 1). YycF bound specifically to these two promoter regions (Fig. 5A and B). The regions protected by YycF binding are located around the previously defined consensus sequence (Fig. 5C). The YycF regulator binds the *isaA* DNA fragment at two distinct regions (Fig. 5A and C). The region with higher apparent affinity for YycF (region I in Fig. 5A and C) contains the direct repeat proposed to be the YycF consensus recognition sequence. The second site (region II in Fig. 5A and C) contains only one-half of the direct repeat, which could explain the lower affinity observed for YycF binding.

DISCUSSION

This report is the first description of genes directly regulated by the essential YycG/YycF TCS in *S. aureus*. Previous work

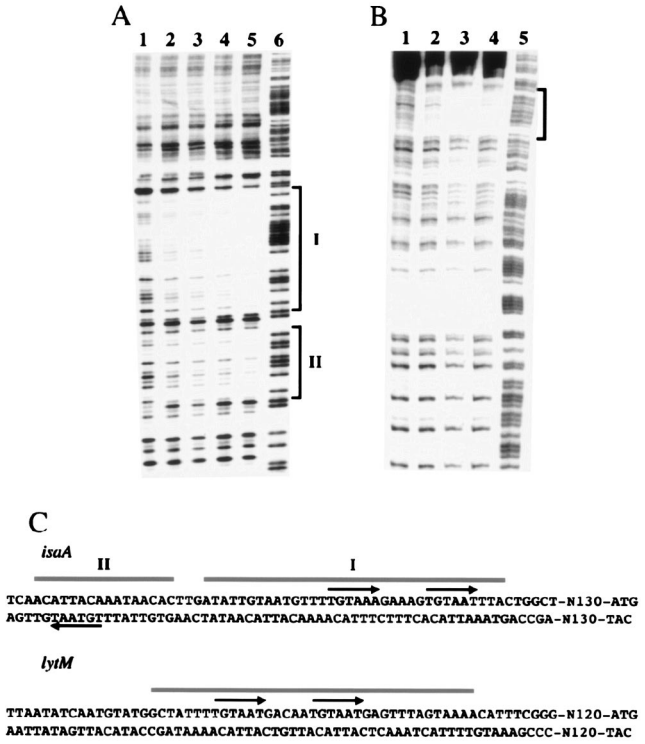

FIG. 5. DNase I footprinting assay of YycF bound to the *isaA* and *lytM* promoter regions. Lanes contain approximately 0.7 pmol (5×10^4 cpm per reaction mixture) of labeled nontemplate strands of *isaA* (−250 to +59 with respect to the translation initiation codon) (A) or *lytM* (−321 to +60 with respect to the translation initiation codon) (B). Fragments were incubated with increasing amounts of purified YycF. (A) Lanes: 1, no protein; 2, 34 pmol; 3, 68 pmol; 4, 136 pmol; 5, 204 pmol; 6, A+G Maxam and Gilbert reaction. (B) Lanes: 1, no protein; 2, 136 pmol; 3, 272 pmol; 4, 408 pmol; 5, A+G Maxam and Gilbert reaction. The brackets indicate regions protected by YycF from DNase I cleavage. (C) Nucleotide sequence of the *isaA* and *lytM* promoter regions. Regions protected by YycF are indicated by bars, and the conserved direct repeats are indicated by arrows.

has highlighted several phenotypes linked to a thermosensitive mutation of the *yycF* locus such as hypersensitivity to MLS_B antibiotics and to unsaturated long-chain fatty acids and partial suppression of the conditional lethal phenotype by high sucrose and NaCl concentrations (27).

Although these characteristics led the authors to suggest that the YycG/YycF TCS could be implicated in regulation of bacterial cell wall or membrane composition, no genes regulated by this system had been identified. A recent report had focused on gene products able to complement the hypersensitivity to MLS_B antibiotics of the conditional *yycF* mutant, and it was suggested that the loss of function or expression of *ssaA* was responsible for this phenotype (26). Although we were not able to reproduce the MLS_B hypersensitivity phenotype with the RN4220P*spac-yycF* mutant (data not shown), specific binding of the purified YycF protein to the *ssaA* promoter region supports the hypothesis of direct regulation of *ssaA* expression. The *ssaA* gene encodes a major antigenic protein, SsaA (staphylococcal secretory antigen), whose function has been investigated in *S. epidermidis* (22). As SsaA from *S. epidermidis* has 74% amino acid sequence identity with SsaA from *S. aureus*, we can postulate that these two proteins have similar functions in these two closely related bacteria. It has been reported that a high rate of anti-SsaA immunoglobulin G antibodies can be detected in sera of patients with *S. epidermidis* endocarditis, suggesting a role for SsaA in the pathogenesis of this disease (22). Interestingly, three genes of unknown function potentially belonging to the YycF regulon of *S. aureus* encode proteins with strong similarities to SsaA (SA0620, SA2097, and SA2353). We have also shown that another important antigenic protein, IsaA (immunodominant staphylococcal antigen), is probably regulated by the YycG/YycF system as well. Although the putative function of this protein is still unknown, the high rate of antibodies raised against IsaA and found during sepsis caused by methicillin-resistant *S. aureus* shows that this protein is expressed at a high rate during infection, which could suggest a putative role in colonizing host tissues (25). Although it has to be verified, three other genes encoding potential virulence factors implicated in interactions with the host extracellular matrix may also be regulated by YycG/YycF. The *sdrD* gene encodes a Ser-Asp-rich sialoprotein-binding protein, a function reported to be important in the pathogenicity of osteomyelitis (41). EbpS is implicated in interactions between *S. aureus* and elastin, one of the major protein components of the extracellular matrix (31, 32). Finally, Sak, the staphylokinase, may have an important physiological function in helping cells to disseminate in host tissue by activating plasminogen (28). A comparison of candidates for YycG/YycF regulation in *B. subtilis* and *S. aureus* reveals several similarities. The sizes of the regulons are quite similar since the *B. subtilis* YycF regulon comprises 14 genes, including 4 genes for which specific binding of YycF has been shown (18), whereas the *S. aureus* YycF regulon is composed of 12 genes, and specific binding was shown for 3 of these. YycF-dependent regulation of *yocH*, which encodes a peptidoglycan hydrolase, has been demonstrated in *B. subtilis* (18). In *S. aureus*, the present work has highlighted binding of YycF to the *lytM* promoter region. LytM is a glycylglycine endopeptidase (33, 34), indicating a likely role for the *S. aureus* YycG/YycF system in controlling cell wall metabolism, as shown for its ortholog in *B. subtilis*. The *yycF* operon is transcribed during exponential growth in *B. subtilis*, and its expression is rapidly shut down as cells enter stationary phase (11). The *yocH* gene follows the same expression profile, and the results suggest that the YycG/YycF system is only active during the exponential growth phase (18). Expression of the *S. aureus yycF* operon has not been studied, but a predicted σ^A promoter was suggested, 215 bp upstream from the *yycF* translation start site, in agreement with expression of the operon during exponential growth (27). This correlates with the *lytM* expression profile since LytM is essentially synthesized during exponential growth (34). The second common link between the *B. subtilis* and *S. aureus* YycF regulons is an oligopeptide ABC transporter. Potential YycF binding sequences are found in front of the *opp* (oligopeptide permease) operons of *B. subtilis* and *S. aureus*, as well as in front of the *oppA* gene of *L. monocytogenes* and the *Streptococcus agalactiae gbs0144* gene, which encodes a protein similar to the binding protein of the Opp system. This suggests that YycF and their orthologs in closely related bacteria could control synthesis of the Opp system. The Opp system has a very pleiotropic role since it is implicated in the uptake of various oligopeptides that act as signals for the cell (for a review, see reference 24). In *B. subtilis*, the Opp system imports extracellular peptides that influence competence and sporulation (20). In *L. monocytogenes*, the Opp system is implicated in bacterial growth at low temperature and intracellular survival (4), whereas it controls extracellular protein production and virulence in *Bacillus thuringiensis* (15).

In summary, our results show that the YycF regulator of *S. aureus* recognizes a tandemly repeated hexanucleotide sequence with a spacer of five nucleotides [5′-TGT(A/T)A(A/T/C)-N_5-TGT(A/T)A(A/T/C)-3′] and that it binds specifically to genes involved in virulence and cell wall metabolism. But the main questions concerning the essential nature of the YycG/YycF system remain unanswered since none of the 12 putative YycF-regulated genes in *S. aureus* have been shown to be essential. In *B. subtilis*, it was suggested that the essential nature of the YycG/YycF system could be due to the regulation of the essential *tagD*/*tagA* genes involved in teichoic acid biosynthesis (18), but we were unable to demonstrate specific binding of YycF to the *tag* operon promoter region of *S. aureus* (data not shown). Two hypotheses could explain the essentiality of the YycG/YycF system. Either it controls several functions that are essential not on their own but only in combination, or it regulates an essential function that was not picked up in our DNA motif analysis. As a complementary approach to identify new genes directly controlled by YycF in *S. aureus*, ChIP-to-chip experiments (chromatin immunoprecipitation followed by hybridization to DNA macroarrays) are now in progress.

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