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Evidence that the Essential Response Regulator YycF in *Streptococcus pneumoniae* Modulates Expression of Fatty Acid Biosynthesis Genes and Alters Membrane Composition†

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The YycFG two-component system, originally identified in *Bacillus subtilis*, is highly conserved among gram-positive bacteria with low G+C contents. In *Streptococcus pneumoniae*, the YycF response regulator has been reported to be essential for cell growth, but the signal to which it responds and the gene members of the regulon remain unclear. In order to investigate the role of YycFG in *S. pneumoniae*, we increased the expression of *yycF* by using a maltose-inducible vector and analyzed the genome-wide effects on transcription and protein expression during the course of *yycF* expression. The induction of *yycF* expression increased histidine kinase *yycG* transcript levels, suggesting an autoregulation of the *yycFG* operon. Evidence from both proteomic and microarray transcriptome studies as well as analyses of membrane fatty acid composition indicated that YycFG is involved in the regulation of fatty acid biosynthesis pathways and in determining fatty acid chain lengths in membrane lipids. In agreement with recent transcriptome data on pneumococcal cells depleted of YycFG, we also identified several other potential members of the YycFG regulon that are required for virulence and cell wall biosynthesis and metabolism.

Two-component signal transduction systems (TCSs) seem to be a fundamental constituent of the regulatory organization in bacteria, and they frequently control the expression of virulence factors and adaptive responses (12; reviewed in reference 3). TCSs typically comprise a histidine kinase (HK) in the membrane that senses an environmental stimulus and a cognate response regulator (RR) in the cytoplasm that controls gene expression through binding to DNA promoter sequences. Signal transduction between the sensor protein and the response regulator is achieved by phosphoryl transfer from a histidine residue in the conserved histidine kinase catalytic site to an aspartate residue in the N-terminal receiver domain of the response regulator. Modulation of the phosphorylation state of the RR by the HK is the basis for their effects on the control of gene expression (23).

In the genome of the human pathogen *Streptococcus pneumoniae*, 13 TCSs have been identified, and among them, only YycFG is essential for this bacterium (27, 41). YycFG homologues are found in several gram-positive pathogens (16), and in *S. pneumoniae*, this system has been designated MicAB (14), VicRK (42), and 492hkrr (41), but it is referred to here as YycFG.

Gene knockout studies of the 34 TCSs in *B. subtilis* showed that YycFG is the only essential TCS. Both the *yycF* and *yycG* genes are required for viability (16), as is also the case for *Staphylococcus aureus* (29). However, for *S. pneumoniae*, only the response regulator *yycF* gene was found to be essential (27, 41), suggesting that YycF functions independently of the adjacent histidine kinase YycG, possibly participating in signal transduction with other TCS histidine kinases.

Although the role of YycFG remains unclear, these proteins may have relevance as novel targets for antimicrobial intervention (38). In *S. aureus*, a point mutation in the *yycF* gene conferred temperature-dependent sensitivity to several macrolide and lincosamide antibiotics and also sensitivity to unsaturated long-chain fatty acids, suggesting that this TCS plays a role in cell permeability or the regulation of cell wall and membrane composition (29). In *B. subtilis*, at a nonpermissive temperature, a conditional null mutant of *yycFG* formed chains of cells, some of them lacking cytoplasmic contents (16), whereas the overexpression of YycF led to the production of minicells and a reduction in the cell length as well as a transcriptional induction of the *ftsAZ* cell division operon (17). Further clues that YycF is important for cell division were provided by a hybrid regulator/transcriptome approach in *B. subtilis* (24). This involved the construction of chimeric response regulators by fusing the receiver domain of a regulator of known function with the DNA binding domain of a regulator of unknown function. Thus, microarray analysis under low-phosphate conditions revealed that a gene (*yocH*) encoding a potential autolysin was induced more than fourfold in the presence of the hybrid PhoP-YycF regulator. The identification of the consensus recognition sequence for YycF, consisting of two hexanucleotide direct repeats separated by five nucleotides, enabled 10 genes involved in cell division, cell wall

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metabolism, and a membrane-bound transport system to be identified as potential members of the YycFG regulon in *B. subtilis*. Similarly, for *S. aureus*, 12 genes potentially regulated by YycF were identified, including the gene for LytM (a peptidoglycan hydrolase), and binding of YycF to this motif was confirmed in vitro (13). However, none of these genes in *S. aureus* are known to be essential.

The functions controlled by YycFG in *S. pneumoniae* are also not fully understood. Mutation of the putative phosphorylation site in the receiver domain of YycF substantially reduced the stability of YycF-PO_4 in vitro, although a clone carrying this mutation did not show altered growth properties compared to the wild-type strain (14). As stated above, the YycG histidine kinase is not essential in *S. pneumoniae*, although a recent study (32) showed that YycG is conditionally required for growth when levels of YycF are reduced by means of a regulatable promoter. In that study, a microarray analysis of mutants with reduced expression of the YycFG TCS showed an altered expression of 49 genes in 10 clusters, encoding proteins of unknown function or proteins involved in transport, heat shock, the cell surface, carbohydrate metabolism, and amino acid biosynthesis. Of these, *pcsB*, encoding a possible extracellular cell wall hydrolase which is also found in other streptococci, may be the essential target of YycF in *S. pneumoniae*, as its constitutive expression suppressed the essentiality of *yycF*.

It was recently shown through a DNA microarray analysis of *B. subtilis* two-component regulatory systems that overproduction of the response regulator of a two-component system affects the regulation of genes controlled by the system (35). In order to further elucidate the role of YycFG in *S. pneumoniae*, we increased the expression of *yycF* by using a maltose-inducible vector, and here we describe the effects on transcription, protein expression, and fatty acid membrane composition, as determined by the use of DNA microarrays, proteomics, and gas chromatography-mass spectrometry.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The *S. pneumoniae* JNR7/87 capsulated strain (40) (The Institute of Genomic Research [http://www.tigr.org]) was used for this work. Cells were grown at 37°C without shaking in AGCH medium (26) supplemented with 0.25% yeast extract (AGCHY) and sucrose and/or maltose at the concentrations indicated in Results. When required, erythromycin was added to the medium at 1 μg ml^{-1} (AGCHYE).

The plasmid pPL100 was constructed as follows. The total genomic DNA from strain R61 was used for PCR amplification with the primers rrNheI (5′-TTGG TATAATAGCTAGCAAAAAGGTGAAC-3′) and hkNheI (5′-AAAATACTG TATTGCTAGCCTATTTCACTC-3′) (NheI restriction sites are underlined) to obtain a 765-bp fragment containing the response regulator *yycF* gene and its ribosomal binding site. The PCR product was then digested with NheI and cloned into the unique XbaI site of the pLS1RGFP expression vector, which contains the *gfp* gene cloned under the control of the pneumococcal P_M promoter and the *malR* gene (33). The resulting plasmid, pPL100, was propagated in *S. pneumoniae* strain R61. Transformants were selected for erythromycin resistance, and the correct nucleotide sequence of the chromosomal insert of pPL100 was confirmed by DNA sequencing. pPL100 was then transferred to the *S. pneumoniae* JNR7/87 capsulated strain by transformation and selection for erythromycin resistance.

Molecular techniques. Standard procedures were used for PCR and cloning procedures. Plasmid DNAs were prepared by the method described by Birnboim and Doly (4), with the modifications described by Stassi et al. (37). Sequencing was performed on an ABI PRISM 320 sequencer (Perkin-Elmer).

Competence and transformation procedures for *S. pneumoniae* strain R6 were performed according to the method of Lacks (26). For *S. pneumoniae* strain JNR7/87, the synthetic competence-stimulating peptide 1 (20) was added to the transformation mixture at a concentration of 25 ng ml^{-1}.

Induction of expression from P_M promoter. Uninduced cultures were grown in AGCHYE medium containing 0.8% sucrose. Frozen cultures were diluted 1:1,000 and grown overnight at 37°C until the cultures reached an optical density at 650 nm (OD_{650}) of 0.4. Overnight cultures were diluted 1:100 in fresh medium. The cultures were maintained in exponential growth phase by suitable dilutions (at least twice for each experiment) in prewarmed medium so that the cell density was always kept below an OD_{650} of 0.4.

Transient induction was performed by a challenge of uninduced cultures with 0.8% maltose for the times indicated in Results. Prior to induction, cells were grown to an OD_{650} of 0.4, harvested by centrifugation at 6,700 × *g* at room temperature, and resuspended in prewarmed fresh AGCHYE medium plus maltose or sucrose.

Long-term induction was performed by growth of the cultures in AGCHYE medium containing 0.8% maltose plus 0.2% sucrose at an OD_{650} of 0.4. Long-term induced cultures were obtained and kept in exponential growth phase as uninduced cultures.

After growth, the cultures were harvested by centrifugation at 6,700 × *g* and washed twice with cold phosphate-buffered saline, pH 8.0 (PBS; 10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl). Pellets were stored at −70°C for protein and RNA extraction.

Measurement of GFP expression. The expression of green fluorescent protein (GFP) in pneumococcal cells was analyzed as previously described (1). Briefly, cells were harvested by centrifugation, washed, and resuspended to the original volume in PBS. Fluorescence was measured with an LS-50B spectrophotometer (Perkin-Elmer), with excitation at 488 nm with a slit of 2.5 and emission at 511 nm with a slit of 10. As a background, the fluorescence of PBS was subtracted from the fluorescence detected in the samples.

Preparation of protein extracts. Frozen pellets from 100-ml cultures were defrosted and resuspended in 4.67 ml of solution 1 (10 mM sodium dodecyl sulfate [SDS], 200 mM dithiothreitol [DTT], 28 mM Tris base, 20 mM Tris-HCl) and 330 μl of solution 2 (24 mM Tris base, 0.47 M Tris-HCl, 50 mM $MgCl_2$, 10 mg of protease-free RNase A [Sigma] ml^{-1}, and 10 mg of protease-free DNase I [Roche] ml^{-1} in 5 mM $CaCl_2$). Total extracts were prepared by passing the cells four times through a French pressure cell at 12,000 lb/in^2. Cell debris was removed by centrifugation at 13,200 × *g* for 15 min. The supernatant was divided into aliquots and stored at −70°C.

The total protein concentrations in the extracts were determined by quantification of the protein bands after separation of the proteins in SDS–12.5% polyacrylamide gels and staining with 0.25% Coomassie brilliant blue. Quantification was performed with Quantity One 4.2.1 software in a Gel Doc 2000 molecular analyst (Bio-Rad Laboratories). The Mark 12 unstained standard (Invitrogen) was used as a reference for the protein amounts. For proteomic analysis, bacterial lysates containing 100 μg of protein were analyzed in individual experiments. The proteins were separated in the first dimension by use of a pHaser system (Genomic Solutions) and gel strips for pHs 4.0 to 7.0 (Amersham-Pharmacia) as recommended by the manufacturers, with minor modifications. The proteins were mixed with lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris base) to a final volume of 50 μl and then with 350 μl of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 2% IPG buffer [pHs 4.0 to 7.0; Amersham-Pharmacia], 18.2 mM DTT, bromophenol blue). The gel strips were rehydrated with the total volume of 400 μl overnight at 21°C. Electrode wicks were put under the ends of the strips, and the strips were covered with dry-strip cover fluid (Amersham-Pharmacia). The gel strips were run for 24 h. The gel strips were rinsed with MilliQ water and equilibrated in filtered equilibration buffer (56% [vol/vol] Tris-acetate equilibration buffer [pH 7.0; Genomic Solutions], 5% SDS, 6 M urea, 30% glycerol) containing 52 mM DTT for 30 min and then in filtered equilibration buffer containing 1.35 M iodoacetamide for 30 min. The proteins were separated in the second dimension in 10% Duracryl gels. The gels were cast according to the manufacturer's protocol (Investigator 2-D electrophoresis system operating and maintenance manual, Genomic Solutions), except that the glass plates were sealed with silicon (ARBO low-modulus silicone sealant; Adshead Ratcliff & Company Limited). The silicon was allowed to dry for at least 1 day. The gels were kept at 4°C for 1 week to 1 month before use. Second-dimension gels were run according to the manufacturer's protocol (Investigator 2-D electrophoresis system operating and maintenance manual) by the use of cathode buffer (0.2 M Tris-HCl, 0.2 M Tricine, 0.4% SDS), anode buffer (25 mM Tris-acetate), and the Investigator system of Genomic Solutions. The gels were stained with Sypro Ruby (Genomic Solutions) according to the manufacturer's instructions. The proXPRESS proteomic imaging system (Perkin-Elmer) was used for imaging.

The protein spots present in the two-dimensional (2D) gels were quantified

with the PDQuest 2D analysis 7.1.0 program (Bio-Rad Laboratories). For this quantification, 136 stained spots were matched in all gels and used for normalization of the average intensity.

Mass spectrometric analysis. The Genomic Solutions Investigator ProPic Robotic Workstation was used to pick spots of interest. Protein spots were washed three times for 20 min each with 100 μl of solution A (freshly prepared 80% 50 mM NH_4HCO_3–20% acetonitrile). The gel plugs were washed with 100 μl of acetonitrile for 10 to 15 min and then air dried for 10 min. Trypsin (5 μl containing 50 ng of trypsin in 10 mM NH_4HCO_3) was added to digest the proteins and incubated at 37°C for 2 to 4 h. The trypsin was inactivated by adding 5% formic acid. The plugs were incubated for 20 min, flash frozen, and stored at −80°C.

For matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis, the spots were analyzed by the John Innes Centre Protein Sequencing Facility. Peptide peak lists were searched against databases of *S. pneumoniae* (Mascot software; Matrix Science).

RNA preparation and semiquantitative reverse transcriptase PCR (RT-PCR). RNAs were prepared from 10-ml cultures by use of a QIAGEN RNeasy Midi kit (QIAGEN) according to the manufacturer's procedure. The total RNA concentrations were determined by UV spectrophotometry. The RNAs were checked for their integrity and yield by analysis in a 1% agarose gel. In addition, the quality and quantity of the RNA samples were checked by use of an RNA 6000 Nano assay with an Agilent 2100 bioanalyzer (Agilent Technologies) according to the manufacturer's protocol.

Semiquantitative analyses of transcript levels of chromosomal *yycFG* were performed by a two-step RT-PCR assay. The primers used were yycFG up (5′-TAGAGCAATTTGAAGCAGAGCAACCAGAT-3′) and yycFG down (5′-ATAAGGGAGTACAATGGTAAAGGTTGAAC-3′). RNAs (400 ng) were added to 20-μl reverse transcription reaction mixes containing 4 μl of cDNA synthesis buffer, 5 mM DTT, 40 U of RNaseOUT (Invitrogen), 1 mM deoxynucleoside triphosphate mix, a 10 μM concentration of the appropriate gene-specific primer, and 15 U of Thermo-Script RT (Invitrogen) and then incubated for 60 min at 60°C. Reverse transcription was terminated by incubation at 37°C for 20 min in the presence of 2 U of RNase H (Invitrogen). For the amplification of *yycFG*, 10% of the cDNA synthesis reaction, 50 pmol of each primer, a 500 mM concentration (each) of dATP, dGTP, dTTP, and dCTP, 3.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), and 50 mM KCl were used for each 50-μl PCR, which was performed with 2 U of *Taq* DNA polymerase (Invitrogen). The cycling conditions were as follows: 1 cycle of 94°C for 4 min and 10, 15, 20, 25, and 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min. Twelve percent of the RT-PCR products were analyzed by 1.5% agarose gel electrophoresis. The intensities of the PCR products were quantified with Quantity One Gel Doc 2000 software (Bio-Rad Laboratories).

Construction of *S. pneumoniae* microarray. DNA fragments of individual open reading frames (ORFs) present in *S. pneumoniae* strain TIGR4 (also known as JNR7/87) and R6 strain-specific genes were amplified by the use of ORF-specific primers (MWG Biotech). The primers (22-mers) were designed according to the approach of Hinds et al. (22), with a predicted melting temperature of 60°C. In total, 2,236 TIGR4 and 117 R6 gene-specific probes were amplified by PCR for spotting onto the microarray. All PCRs were performed with HotStart *Taq* (QIAGEN) and a Primus-HT PCR machine (MWG Biotech) according to the following parameters: 95°C for 15 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 120 s, with a final incubation at 72°C for 600 s. Genomic TIGR4 and R6 DNAs (2.5 ng) were used as templates, and approximately 100 pmol of each primer was used in a reaction volume of 50 μl. For each primer pair, the successful PCR amplification of a single DNA fragment of the correct length was checked by electrophoresis in agarose gels. In the initial high-throughput PCR, a small percentage of reactions (approximately 5%) were either unsuccessful or gave poor yields of product. However, these missing probes were obtained subsequently by optimizing the PCR conditions or by designing new primers. Ten probes carrying ORFs from *Campylobacter jejuni* with no significant DNA sequence homology to *S. pneumoniae* were also included on the array as negative controls. All PCR products were purified by the use of SigmaSpin 2 postreaction clean-up plates (Sigma), dried, and resuspended in half the original volume in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.01% Sarkosyl before being spotted onto the microarray. PCR products were spotted onto GAPS II-coated slides (Corning) by use of an in-house Stanford-designed array machine (see http://cmgm.stanford.edu/pbrown/mguide for associated software and protocols). Each glass slide contained two arrays, with each containing two probes (or features) for each gene.

Fluorescent labeling of RNA and microarray hybridization. Fifteen micrograms of total purified RNA was mixed with 5 μg of random primers and Sigma ultrapure water to a final volume of 12.4 μl. The following components were added: 3 μl of 10× RT buffer (Stratagene), 0.6 μl of 50× deoxynucleoside triphosphates (25 mM [each] dATP, dGTP, and dTTP and 10 mM dCTP), 3 μl of 0.1 M DTT, 2 μl of Cy3 or Cy5 (Amersham-Pharmacia), and 4 μl of RT (Stratagene). The reaction mixtures were incubated overnight at 37°C. Reverse transcription reactions were stopped by the addition of 1.5 μl of 20 mM EDTA, pH 8.0, and 15 μl of freshly made 0.1 M NaOH and by incubation at 70°C for 15 min. Reactions were neutralized by adding 15 μl of 0.1 N HCl before purifying the cDNAs with a PCR purification kit (QIAGEN). Eluted cDNAs were concentrated in a Savant SpeedVac and then resuspended in a solution containing 25 μl of Sigma ultrapure water, 3 μl of human Cot1 DNA (Invitrogen), 6 μl of 20× SSC, 1 μl of 1 M HEPES (pH 7.0), 1 μl of 10% SDS, and 4 μl of 50× Denhardt solution. Hybridization reactions were incubated in boiling water for 2 min and then allowed to cool at room temperature for 10 min before being centrifuged at maximum speed in a bench-top centrifuge for 2 min. The supernatants were carefully removed and placed into fresh tubes, and 40 μl was pipetted onto an array. Hybridization reactions were covered with a coverslip (Sigma Hybri-slip) and placed in a hybridization chamber (Gene Machine). Hybridization reactions were incubated overnight at 62°C. Following hybridization, the slides were washed twice for 5 min each in wash buffer 1 (2× SSC, 0.1% SDS), twice in wash buffer 2 (1× SSC), and twice in wash buffer 3 (0.2× SSC) before being dried by centrifuging at 50 × *g* for 5 min.

Microarray data analysis. Microarray data analysis was conducted on RNAs purified from three independent cultures of each strain. Microarrays were scanned by use of an Axon 4000A microarray scanner, and images were acquired with GenePixPro 3.0 software (Axon). The median feature and background intensities for each feature were imported into GeneSpring 6.0 (Silicon Genetics). Data were normalized by intensity-dependent normalization (LOWESS [9]), and the cross-gene error model was based on replicate measurements. Gene lists were prepared from the genes outside a 1.75-fold cutoff. Gene lists were filtered by rejecting genes with *P* values (derived from one-sample Student's *t* test calculated within GeneSpring) of >0.01, with the exception of genes predicted to be cotranscribed based on a computational prediction of operons (15). Gene lists were further filtered to remove genes that were affected by the expression system, as determined by analyzing microarray data derived from hybridizations with RNAs extracted from strain JNR7/87(pLS1RGFP) grown in the presence or absence of maltose at 5 min and under long-term conditions. The results obtained with this method were in agreement with those obtained by use of an in-house expression analysis tool similar to that described by Pearson et al. (36).

Analysis of membrane fatty acid composition. For determinations of the fatty acid composition of the membrane, cells of *S. pneumoniae* JNR7/87(pLS1RGFP) and JNR7/87(pPL100) frozen cultures were diluted 1:1,000 in AGCHYE medium containing 0.8% sucrose and grown overnight at 37°C until reaching an OD_{650} of 0.4. Overnight cultures were diluted 1:100 in NOCH medium containing 0.8% maltose, 0.2% sucrose, and erythromycin at 1 μg ml^{-1}. In this AGCH modified medium, the vitamin-free Casamino Acids were replaced with the following amino acids, each at 50 μg ml^{-1}: Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Cys. The casein hydrolysate and serum albumin were reduced, respectively, to 0.05 and 0.01%. This modification was required to minimize the incorporation of fatty acids present in the medium into the pneumococcal membrane. The cultures were maintained in exponential growth phase by dilution twice in fresh medium, keeping the cell density below 0.4. After growth, the cultures were harvested and the pellets were washed with PBS. Total cellular fatty acids were prepared by the method of Bligh and Dyer (5). The fatty acid methyl esters were prepared by the transesterification of glycerolipids with 0.5 M sodium methoxide in methanol (8) and then were analyzed in a capillary column (30 m by 0.25-mm internal diameter) of 100% dimethylpolysiloxane (PE-1; Perkin-Elmer) by use of a Perkin-Elmer Turbo Mass gas chromatograph-mass spectrometer. Helium at 1 ml min^{-1} was used as the carrier gas, and the column was programmed to ramp at 4°C min^{-1} from 140 to 240°C. Straight-chain fatty acids and unsaturated fatty acids used as reference compounds were obtained from Sigma Chemical Co. The positions of the double bonds in these unsaturated fatty acids were determined by gas chromatography-mass spectrometry. Fatty acid methyl esters were converted to dimethyl disulfide adducts as described previously (44) and then separated in a PE-1 column ramped from 140 to 280°C at 4°C min^{-1}. The spectra were recorded in the electron impact mode at 70 eV, with 1-s scans of *m*/*z* 40 to 400.

RESULTS

Construction of *S. pneumoniae* strain JNR7/87(pPL100) and inducible expression of the response regulator YycF-encoding gene. To construct a vector for the inducible expression of

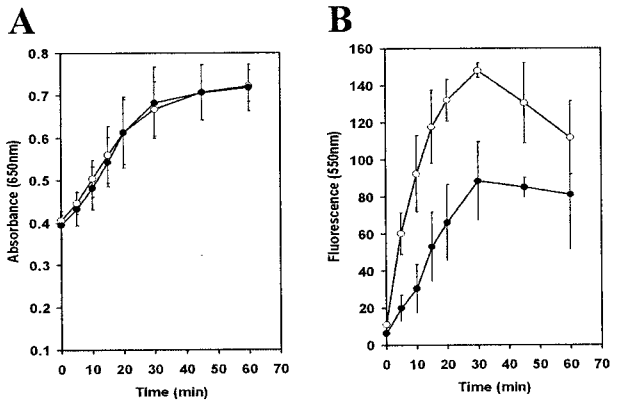


FIG. 1. Growth curve analysis and detection of GFP fluorescence. Cultures of *S. pneumoniae* JNR7/87(pPL100) (●) and JNR7/87(pLS1RGFP) (○) in exponential growth phase were induced with maltose at time point zero. The optical densities of the cultures were measured over time (A). GFP fluorescence was also monitored over time by use of a spectrophotometer (B).

yycF, we clone the gene between the P_M promoter and the *gfp* reporter gene in the maltose-inducible expression vector pLS1RGFP (33) (see the details of construction in Materials and Methods). In this construct, the downstream *gfp* gene serves as a reporter to monitor inducible promoter activity and the expression of *yycF*. In addition, the vector encodes the MalR transcriptional regulator (34), which represses expression from the P_M promoter when maltose is not present in the growth medium. The resulting plasmid, pPL100, was transformed into the capsulated strain *S. pneumoniae* JNR7/87 to create JNR7/87(pPL100). Similarly, JNR7/87 carrying the vector pLS1RGFP [i.e., strain JNR7/87(pLS1RGFP)] was constructed to control for the effects of maltose induction and is universally referred to as the control strain in this study.

Maltose induction did not adversely affect the growth of JNR7/87(pPL100) compared to the control strain over a 60-min period of induction in exponential phase (Fig. 1A). As expected, maltose induction resulted in the expression of GFP in both the JNR7/87(pPL100) and control strains, as shown by spectroscopy (Fig. 1B). Although GFP fluorescence was lower in JNR7/87(pPL100) cells than in control cells, the pattern of GFP expression over time was similar for both strains. Maltose induction resulted in an increase in GFP fluorescence within 5 min, which reached a peak level at 30 min postinduction. The continued growth of JNR7/87(pPL100) in medium containing 0.8% maltose as the sole carbon source substantially impaired growth compared to the growth of this strain in normal medium, presumably due to the effects of the accumulation of YycF. The addition of 0.2% sucrose to the induction medium reduced this adverse effect, and both strains remained in the exponential growth phase to an OD_{650} of 0.5 or higher. This long-term induction condition (medium containing 0.8% maltose and 0.2% sucrose) was used for further experiments, although cell growth was still affected in JNR7/87(pPL100) (doubling time, 40.0 ± 6.2 min) compared to JNR7/87(pLS1RGFP) (doubling time, 23.1 ± 1.7 min).

Transcriptional analysis of *S. pneumoniae* after induction of *yycF*. Whole-genome microarray comparisons of RNA transcript levels were performed for strain JNR7/87(pPL100) and the control strain 5 min after induction and after long-term induction. Three separate cultures were used for biological replicates, and on average, data were obtained for 12 features per gene. A complete list of genes with affected transcript levels by YycF induction and their functional classifications after short- and long-term induction are given, respectively, in Table 1 and in Table S1 in the supplemental material. As expected, increased transcript levels of the *yycF* gene (Sp1227) were detected in strain JNR7/87(pPL100), but not in the control strain, after induction. Apart from this, the major effects on gene transcription were restricted to functions influencing the membrane composition and transport. For example, the expression of 12 of 13 adjacent genes of the fatty acid biosynthetic cluster (Fig. 2B) was significantly altered in strain JNR7/87(pPL100) compared to the control (Fig. 2C), with the transcript levels of *fabK*, *fabD*, *fabG*, *fabF*, *accB*, *fabZ*, *accC*, *accD*, and *accA* (Sp0419 to Sp0427) being increased (Fig. 2C). Decreased expression of the *piaBCDA* (Sp1869 to Sp1872) gene cluster, which is involved in iron transport (also called *pit1BCDA*) and is required for virulence in vivo (6, 7), was observed in JNR7/87(pPL100) after short-term induction (Table 1). This result correlates with the induction of this gene cluster by the depletion of YycF (32). Also, the overproduction of YycF resulted in an increase or reduction of transcription of genes encoding transporters (Table 1; also see Table S1 in the supplemental material). Interestingly, the expression of a number of genes with functions related to cell wall biosynthesis and metabolism was affected in response to the long-term overexpression of *yycF* (see Table S1 in the supplemental material). The endo-beta-*N*-acetylglucosamidase gene *lytB* (Sp0965); the essential gene *pscB* (Sp2216), encoding a putative cell wall hydrolase (32); SP2021, encoding a putative glycosyl hydrolase; and two other genes containing a LysM-encoding domain (Sp0107 and Sp2063) were expressed at higher levels in strain JNR7/87(pPL100) than in the control.

In addition, the overproduction of YycF also resulted in increased levels (5.5- and 7.1-fold after short- and long-term induction, respectively) of the cognate histidine kinase (*yycG*) transcript (Sp1226), which in *S. pneumoniae* R61, is cotranscribed with *yycF* (Sp1225) (32, 42). The induction of the chromosomal *yycFG* genes was verified by a semiquantitative RT-PCR with one set of primers spanning the *yycF* and *yycG* genes. RNA samples prepared from JNR7/87(pPL100) and the control strain before and after 10- and 30-min inductions with maltose were reverse transcribed by use of a primer specific to the chromosomally located *yycG* gene to avoid reverse transcription of the *yycF* transcripts that originated from the plasmid. After reverse transcription and removal of the RNA, the bicistronic cDNA encoding YycF and YycG was amplified by PCR (Fig. 3). Quantification of the amplified DNA fragments detected after 20, 25, and 30 PCR cycles revealed that before induction (time zero), the levels of the chromosomal *yycFG* transcript were similar (1.20- ± 0.07-fold variation) in both strains analyzed. However, after 10 and 30 min of induction, the levels of the *yycFG* mRNA were 2.17- ± 0.26- and 4.10- ± 0.42-fold higher, respectively, in JNR7/87(pPL100) than in JNR7/87(pLS1RGFP). In addition, induction for 10 and 30 min resulted in 1.61- ± 0.03- and 2.84- ± 0.03-fold increased levels of the TCS transcript in strain JNR7/87(pPL100), but not in the vector control strain. These results support the

TABLE 1. Genes with significantly different transcript levels in JNR7/87(pPL100) and JNR7/87(pLS1RGFP) cells after short-term maltose induction

Functional category	TIGR4 no.	Gene product (gene)	Ratio[a]	*P* value
Fatty acid metabolism	Sp0419	Enoyl-(acyl carrier protein) reductase (*fabK*)	1.965	9.67E−08
	Sp0420	Malonyl CoA-acyl carrier protein transacylase (*fabD*)	2.022	4.15E−06
	Sp0421	3-Oxoacyl-[acyl carrier protein] reductase (*fabG*)	1.893	1.73E−03
	Sp0422	3-Oxoacyl-(acyl carrier protein) synthase II (*fabF*)	1.796	5.93E−05
	Sp0423	Acetyl-CoA carboxylase, biotin carboxyl carrier protein (*accB*)	1.855	1.96E−03
	Sp0424	Hydroxymyristoyl-(acyl carrier protein) dehydratase (*fabZ*)	2.027	1.04E−06
	Sp0425	Acetyl-CoA carboxylase, biotin carboxylase (*accC*)	2.007	5.51E−07
	Sp0426	Acetyl-CoA carboxylase, carboxyl transferase, β subunit (*accD*)	1.861	7.74E−06
	Sp0427	Acetyl-CoA carboxylase, carboxyl transferase, α subunit (*accA*)	2.098	1.06E−05
Regulation	Sp1226[b]	Histidine kinase (*yycG*)	5.466	1.39E−10
	Sp1227[b]	Response regulator (*yycF*)	3.366	3.24E−05
	Sp1799	Sugar-binding transcriptional regulator, LacI family	0.541	9.19E−02
Transport	Sp0042	Competence factor transporting permease protein ComA (*comA*)	1.992	1.25E−03
	Sp1682	Sugar ABC transporter, permease protein	0.556	1.98E−02
	Sp1683	Sugar ABC transporter, sugar-binding protein	0.469	2.53E−03
	Sp1684	PTS system, IIBC components	0.440	7.03E−03
	Sp1869[b]	Iron ABC transporter, permease protein (*piaB*)	0.514	8.75E−04
	Sp1870[b]	Iron ABC transporter, permease protein (*piaC*)	0.535	8.92E−03
	Sp1871[b]	Iron ABC transporter, ATP-binding protein (*piaD*)	0.538	5.87E−04
	Sp1872[b]	Iron ABC transporter, iron binding protein (*piaA*)	0.579	5.49E−02
	Sp1884	Trehalose PTS system, IIABC components	0.264	9.00E−05
Energy metabolism	Sp1883	Dextran glucosidase DexS, putative	0.392	1.24E−02
	Sp2148	Arginine deiminase (*arcA*)	0.303	2.11E−04
	Sp2150	Ornithine carbamoyltransferase (*argF*)	0.419	7.19E−04
	Sp2166	L-Fuculose phosphate aldolase (*fucA*)	0.533	5.06E−02
	Sp2167	L-Fuculose kinase fucK, putative	0.328	6.09E−03
Hypothetical	Sp0125	Hypothetical protein	2.355	3.30E−04
	Sp1685	Conserved hypothetical protein	0.461	1.90E−02

[a] Genes with >1.75-fold expression changes between JNR7/87(pPL100) and JNR7/87(pLS1RGFP) were selected.
[b] Also detected by Ng et al. after depletion of YycF (32).

observation that the promoter for the chromosomal operon is induced by increased expression of *yycF* carried by the plasmid pPL100. Reactions containing no RT were included in the analysis to confirm the absence of DNA contamination.

Proteomic analysis of *S. pneumoniae* after YycF overproduction. To investigate the proteomic response of *S. pneumoniae* to *yycF* induction, we harvested JNR7/87(pPL100) and the control strain for protein extraction after induction for 5 or 30 min and after long-term induction. Three independent samples were analyzed by 2D gel electrophoresis, Sypro Ruby staining, and fluorescence imaging. Representative images containing approximately 400 distinct protein spots are shown in Fig. 4 for JNR7/87(pPL100) and the control strain for long-term induction. Differences in protein expression were quantified with PDQuest image analysis software. Nineteen protein spots with altered expression in JNR7/87(pPL100) compared to the control (indicated in Fig. 4) were excised from the gels, digested with trypsin, and identified by MALDI-TOF MS using the protein mass fingerprint technique and the Mascot search tool (Matrix Science). The identified proteins, their predicted functions, and their changes in expression level, as judged by the quantification of protein fluorescence, are summarized in Table 2.

Only one protein, the Sp1804 product, was reduced in JNR7/87(pPL100) compared to the control at all times after induction. This protein is a homologue of protein 24 (Gls24) of *Enterococcus faecalis*, and in that host is involved in general stress resistance (19).

As anticipated, an increased amount of the YycF protein was detected in JNR7/87(pPL100) at all time points postinduction. Two protein spots corresponding to the response regulator were identified (spots indicated with "RR*" and "RR" in Fig. 4B) in JNR7/87(pPL100). The more positively charged spot (RR) was more abundant than the more negatively charged protein spot (RR*) (3.3-, 6.9-, and 3.4-fold more abundant after 5-min, 30-min, and long-term induction). The increase in RR levels (reaching the maximum after 30 min of induction) was similar to that observed for GFP (Fig. 1B and data not shown).

At all time points postinduction, 18 protein spots (including the two isoforms of RR) were increased in JNR7/87(pPL100) compared to the control strain (Table 2). These were identified as seven proteins involved in fatty acid biosynthesis (AccABC and FabKDGF, encoded by Sp0419 to Sp0425 and Sp0427), the translation elongation factor Tu (Sp1489 gene product), four proteins involved in purine biosynthesis (PurCMHB, encoded by Sp0044, Sp0047, Sp0050, and Sp0056), the formate tetrahydrofolate ligase (FTHFL, the Sp1229 gene product), which generates a substrate for PurH, and one protein involved in pyrimidine biosynthesis (CarB, the Sp1275 gene product). Fifty percent of these proteins were also found by transcriptome analysis after the short-term induction of *yycF*. The regulatory protein for the pyrimidine operon (PyrR, the Sp1278 gene product) and the ribonucleoside-diphosphate reductase 2 β subunit (NrdF, the Sp1180 gene product), which is involved in purine ribonucleotide biosynthesis, were also increased in

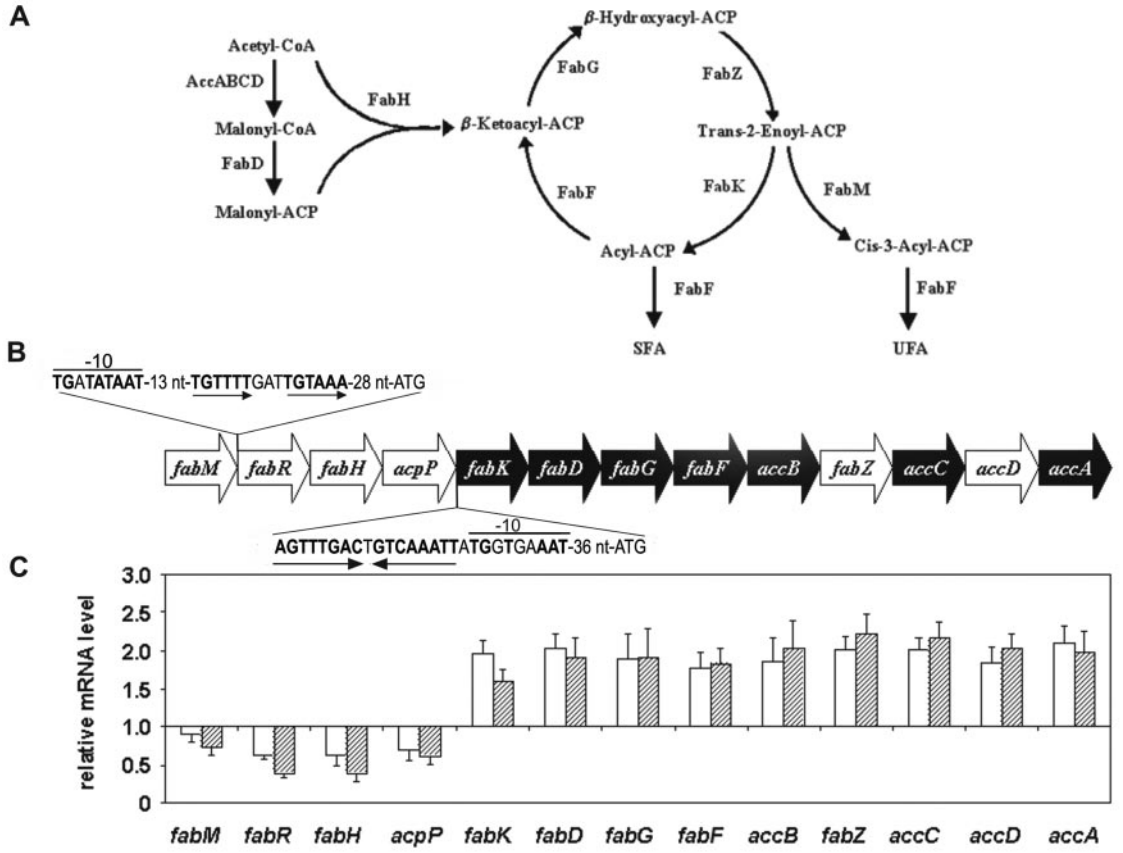


FIG. 2. Fatty acid biosynthesis in *S. pneumoniae*. (A) Pathway of saturated (SFA) and unsaturated (UFA) fatty acid biosynthesis in pneumococci. (B) Pneumococcal biosynthetic gene cluster. Black arrows indicate genes with increased protein levels, as detected by proteomic analysis. Putative promoters for *fabR*, *fabH*, and *acpP* and for *fabK*, *fabD*, *fabG*, *fabF*, *accB*, *fabZ*, *accC*, *accD*, and *accA* as well as putative operators for YycF (two hexanucleotide direct repeats) and FabR (one inverted repeat) are depicted. Nucleotides matching consensus sequences are shown in bold. (C) Relative mRNA expression levels of fatty acid biosynthesis genes in strain JNR7/87(pPL100) compared to the control strain after 5-min (white bars) and long-term (shaded bars) induction. The lengths of the error bars represent the standard deviations from the means.

JNR7/87(pPL100), but only 5 and 30 min after induction, not during long-term induction.

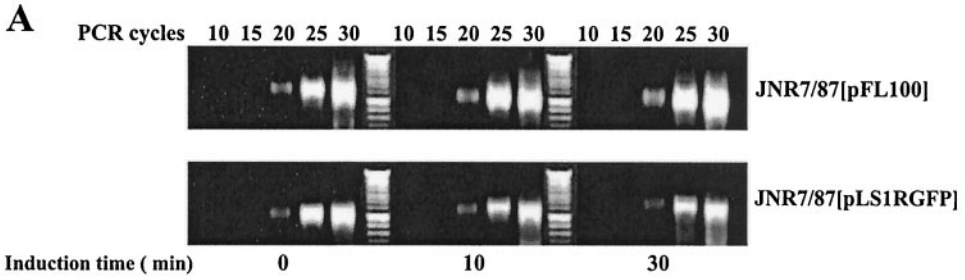
The increased levels of PurCMHB as well as of CarB and PyrR (Table 2) correlated with the microarray analysis of cells grown in the presence of maltose, by which the increased transcription of genes included in the Pur (*purC* and *vanZ*) as well as in the Pyr (*carBA* and *pyrB*) operon was detected (results not shown). These data suggest that the overexpression of *yycF* results in an increase in mRNA synthesis, probably devoted to protein synthesis since increased levels of the elongation factor Tu were also detected in the YycF overproducer strain.

Effects of *yycF* induction on membrane fatty acid composition. Both the proteomic and transcriptome analyses pointed to an increase in expression of the fatty acid biosynthetic genes *fabK*, *fabD*, *fabG*, *fabF*, *accB*, *fabZ*, *accC*, *accD*, and *accA* (Sp0419 to Sp0427) after the induction of *yycF*, suggesting that there may be a change in the composition of the membrane. Accordingly, the fatty acid membrane composition of the control and JNR7/87(pPL100) strains were analyzed by gas chromatography-mass spectrometry after growth in medium containing 0.2% sucrose and 0.8% maltose. The results are depicted in Table 3. The *S. pneumoniae* membrane consists primarily of straight-chain, 16- and 18-carbon saturated and unsaturated fatty acids (28). In the control strain, the ratio of 18-carbon fatty acids ($C_{18:0}$, $C_{18:1\Delta9}$, $C_{18:1\Delta11}$, and $C_{18:1\Delta13}$) to 16-carbon fatty acids ($C_{16:0}$, $C_{16:1\Delta7}$, $C_{16:1\Delta9}$, and $C_{16:1\Delta11}$) was 1.16. However, the strain overexpressing *yycF* produced a relatively larger proportion of C_{18} fatty acids, increasing the total C_{18}/C_{16} ratio to 1.83. A lower ratio of C_{18} to C_{16} fatty acids was also observed for the control strain when the unsaturated and saturated forms of these fatty acids were compared separately (Table 3). The ratios of total unsaturated to saturated fatty acids were very similar for the control and JNR7/87(pPL100) strains, calculated as 1.10 and 1.12, respectively. These data therefore support the conclusion that the overproduction of YycF ultimately impacts the membrane composition.

DISCUSSION

We have investigated the role of the response regulator protein YycF in *S. pneumoniae* by proteome and transcriptome analyses over time following the induction of *yycF* expression. To do this, we cloned *yycF* and a downstream *gfp* reporter gene downstream of the maltose-inducible promoter P_M in plasmid pPL100, which was then transformed into *S. pneumoniae*

A



B

cDNA	Time of induction	PCR cycles	Intensity (arbitrary units)	Ratio (pPL100/vector)
pPL100	0	20	13.9	1.12
pPL100	0	25	83.9	1.18
pPL100	0	30	123.0	1.29
vector	0	20	12.4	
vector	0	25	70.8	
vector	0	30	95.4	
pPL100	10	20	22.9	2.39
pPL100	10	25	134.0	1.80
pPL100	10	30	194.0	2.31
vector	10	20	9.6	
vector	10	25	74.6	
vector	10	30	83.9	
pPL100	30	20	43.3	3.64
pPL100	30	25	262.0	4.65
pPL100	30	30	282.0	4.00
vector	30	20	11.9	
vector	30	25	56.4	
vector	30	30	70.5	

FIG. 3. Semiquantitative RT-PCR analysis of the *yycFG* genes. (A) Agarose gel analysis of RT-PCR products from RNAs extracted from exponential cultures of JNR7/87(pPL100) and the control strain grown with sucrose (time zero) or induced with maltose for 10 and 30 min. PCRs were performed for 10, 15, 20, 25, and 30 cycles. (B) Quantification of RT-PCR products shown in panel A. The intensities of the bands were measured, and ratios were calculated by dividing the intensity values of RT-PCR products from JNR7/87(pPL100) (pPL100) and JNR7/87(pLS1RGFP) (vector) at each induction time and each number of PCR cycles.

JNR7/87. The induction of YycF expression from P_M in JNR7/87(pPL100) was confirmed by an increase in fluorescence due to GFP, by the increased amounts of YycF detected on 2D protein gels, and by higher *yycF* transcript levels (2.9- to 4.4-fold) than those in the control strain.

The two YycF proteins that were identified on 2D gels from extracts derived from induced JNR7/87(pPL100) were not detected in the control strain, indicating that this response regulator is normally present in small amounts during in vitro culture. This is in agreement with the work of Wagner et al. (42), who showed that the transcript of the *yycF* gene was not detected by Northern blotting with an RNA prepared from *S. pneumoniae* cells grown to exponential or early stationary phase. Based on the pIs of the two YycF proteins of JNR7/87(pPL100), we suggest that the more negatively charged spot is the phosphorylated form of the regulator. Notably, the more positively charged spot of YycF was present in larger amounts (3.3- to 6.9-fold) than the more negatively charged species. The induction of YycF resulted in increased transcription of the chromosomal cognate sensor kinase gene (*yycG*), which is located in the same operon (42). A semiquantitative PCR analysis of transcripts encoding YycFG in the control and *yycF*-overexpressing strains (Fig. 3) confirmed that the transcription of both genes was induced upon YycF overproduction. These results indicate that the response regulator autoregulates the expression of the YycFG TCS system. The increased expression of YycG histidine kinase may be responsible for the presence of the putative phosphorylated YycF detected on 2D gels. However, an interaction between YycF and other histidine kinases present in pneumococci cannot be ruled out, since it has been shown that YycF can act as a phosphoacceptor for a heterologous histidine kinase from the *E. faecalis* VanR/VanS TCS in vitro (42). The same authors showed that in *S. pneumoniae*, *yycF* is cotranscribed with the downstream cognate histidine kinase *yycG* gene.

Proteomic studies revealed that 17 proteins were increased in strain JNR7/87 when YycF was overproduced (Table 2). The relative transcript levels of a similar number of genes (11) were also elevated at 5 min postinduction of *yycF* (Table 1). For a variety of reasons (e.g., protein solubility, the sensitivity of techniques, and translational control), the results of proteomic and microarray transcription analyses were expected to be different, but nevertheless, 50% of the affected proteins were identified by both techniques, giving credence to the results.

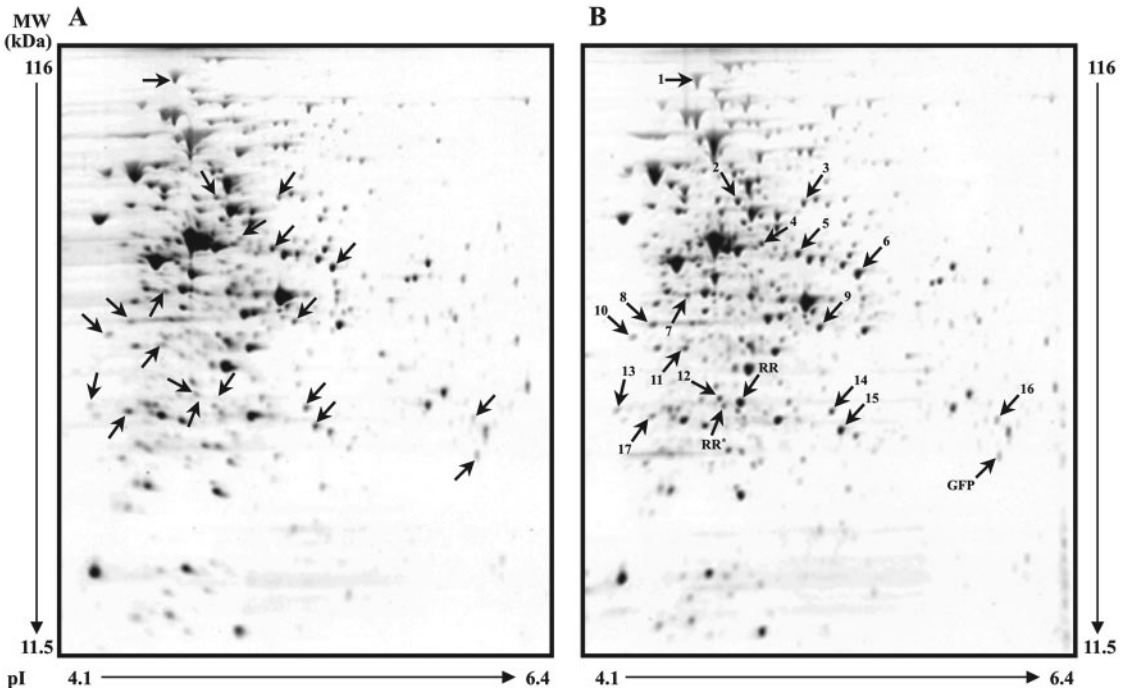


FIG. 4. Proteomic analysis of long-term maltose induction of *S. pneumoniae* strains JNR7/87(pLS1RGFP) and JNR7/87(pPL100). Protein extracts of long-term induced JNR7/87(pLS1RGFP) (control strain) (A) and JNR7/87(pPL100) (B) cultures were analyzed by 2D gel electrophoresis as described in the text. Arrows indicate polypeptides that were present in increased and decreased amounts in JNR7/87(pPL100) compared to the control. The numbers correspond to those used for the proteins listed in Table 2. The GFP spot is also indicated.

The most interesting aspect of our studies was that the proteomic and transcription profiling data provided evidence that the YycFG TCS directly or indirectly controls the expression of the fatty acid biosynthetic pathway (Fig. 2A). Proteomics showed that seven proteins (FabK, FabD, FabG, FabF, and AccBCA) from the fatty acid biosynthesis cluster were present in significantly higher levels in strain JNR7/87(pPL100) than in the control strain after induction. This was corroborated by the microarray transcriptome data, which showed that the expression of the genes for these seven proteins was also increased, and additionally, there was increased expression of *fabZ* and *accD*. The transcriptome data also showed a reduction of expression of *fabR*, *fabH*, and *acpP* after the induction of *yycF* (Fig. 2C). In bacteria, fatty acid biosynthesis is performed by a set of individual enzymes known as the type II or dissociated fatty acid synthase system (reviewed in reference 21). Chains of 16- and 18-carbon saturated and monounsaturated fatty acids are the main end products of the pathway and constitute the hydrophobic portions of the membrane phospholipids.

The FabK, FabG, FabZ, and FabF proteins are all involved in the repetitive series of processes that are necessary for the sequential addition of malonyl-coenzyme A (CoA) molecules to the growing hydrocarbon chain during fatty acid biosynthesis (during this series of events, the malonate is decarboxylated, and thus the chain elongates two carbons at a time). The AccABCD proteins form a multisubunit enzyme responsible for the synthesis of malonyl-CoA from acetyl-CoA. Thus, all of the proteins associated with fatty acid chain elongation were overproduced, at the protein and/or transcript level, in JNR7/87(pPL100). However, *acpP* encodes the acyl carrier protein (ACP), which is the fundamental starting point for fatty acid biosynthesis. FabH is responsible for condensing acetyl-CoA with malonyl-ACP to form β-ketobutyryl-ACP, which is the starter molecule for subsequent chain elongation. FabR is a DNA binding protein belonging to the MarR family of transcriptional regulators (2) and may play a regulatory role in the production of fatty acid elongation genes (see below). *fabR*, *fabH*, and *acpP*, all showed a decrease in transcription in JNR7/87(pPL100) after the induction of *yycF*. Therefore, it appears that genes involved in fatty acid starter unit production have reduced expression after *yycF* induction, whereas those involved with chain elongation have elevated expression. This metabolic imbalance may explain why the C_{18} to C_{16} fatty acid ratio (Table 3) was increased in JNR7/87(pPL100) compared to the control strain.

It has been well established that a lipid bilayer that is completely in a liquid crystalline state has the best prerequisites for maintaining the integrity and the permeability barrier of the membrane and for supporting the full activity of membrane-associated enzymes and transport proteins (31). However, the membrane fluidization temperature has been shown to increase with the effective chain length, which is proportional to the thickness of the well-packed hydrocarbon region (10). Our results indicate that YycF plays a role in determining the membrane composition, and it seems plausible that the unbalanced synthesis of long-chain fatty acids contributes to the abnormal septation observed with strain JNR7/87(pPL100) (results not shown), as it has been reported that the degree of membrane fluidity seems to be important for cell division site selection (30).

TABLE 2. Proteins affected by YycF induction, as detected by proteomic analysis

Functional2 category	Protein[a]	Spot no.	Ratio of induction[b]			TIGR4 no.	Score (%)[c]	Coverage (%)[d]	pI	Molecular mass (kDa)
			5 min	30 min	Long-term					
Fatty acid metabolism	FabK[e]	9	2.25	2.20	2.00	Sp0419	110	46	5.12	34.156
	FabD[e]	11	1.56	1.91	1.40	Sp0420	95	38	4.45	33.184
	FabG[e]	15	2.39	2.57	1.84	Sp0421	207	68	5.34	25.739
	FabF[e]	6	1.49	1.74	1.75	Sp0422	52	13	5.45	44.075
	AccB[e]	13	2.05	2.02	1.93	Sp0423	53	32	4.17	17.023
	AccC[e]	4	1.34	1.44	1.57	Sp0425	123	34	4.85	49.793
	AccA[e]	16	2.74	2.51	1.80	Sp0427	73	18	6.31	28.230
Regulation	RR[e]	RR	>16.20	>39.22	>33.08	Sp1227	171	42	4.78	24.265
	RR*	RR*	>4.90	>5.65	>9.75	Sp1227	207	48	4.71	23.251
Purine and pyrimidine biosynthesis	PurC[e]	12	1.22	1.57	1.87	Sp0044	220	66	4.64	27.043
	PurM	7	1.32	1.15	1.24	Sp0047	95	27	4.45	36.502
	PurH	2	1.39	1.21	1.62	Sp0050	162	28	4.75	56.316
	PurB	5	1.39	1.65	1.17	Sp0056	249	54	5.03	49.617
	CarB[e]	1	1.32	1.35	1.51	Sp1275	224	27	4.52	116.499
	PyrR	14	1.29	1.39	0.99	Sp1278	158	56	5.16	19.597
	NrdF	10	1.24	1.61	0.98	Sp1180	117	37	4.18	36.961
Central intermediary metabolism	FTHFL	3	1.42	1.81	1.65	Sp1229	134	25	5.09	59.806
Protein synthesis	EF Tu	8	1.98	1.86	1.30	Sp1489	197	42	4.60	43.943
Stress	Gls24	17	0.76	0.61	0.43	Sp1804	200	69	4.33	21.803

[a] AccA, acetyl-CoA carboxylase carboxyl transferase alpha subunit; AccB, acetyl-CoA carboxylase biotin carboxyl carrier protein; AccC, acetyl-CoA carboxylase biotin carboxylase; CarB, carbamoyl-phosphate synthase, large subunit; EF-Tu, translation elongation factor Tu; FabD, malonyl CoA-acyl carrier protein transacylase; FabF, oxoacyl-(acyl carrier protein) synthase II; FabG, 3-oxoacyl-(acyl carrier protein) reductase; FabK, enoyl-(acyl carrier protein) reductase; FTHFL, formate tetrahydrofolate ligase; Gls24, hypothetical general stress protein 24; NrdF, ribonucleoside diphosphate reductase 2, β subunit; PurB, adenylosuccinate lyase; PurC, phosphoribosyl-aminoimidazole succino-carboxamide synthase; PurH, phosphoriboxyl- aminoimidazole-carboxamide-formyltransferase/IMP cyclohydrolase; PurM, phosphoribosylformylglycinaamide cycloligase; and PyrR, pyrimidine operon regulatory protein.

[b] Ratios were calculated by dividing the mean values of the spot intensities (from three independent experiments) obtained for JNR7/87(pPL100) and JNR7/87 (pLS1RGFP). The mean values and standard deviations of the spot intensities are depicted in Table S2 in the supplemental material.

[c] The score is $-10\log(P)$, where P is the probability that the observed match is a random event.

[d] Coverage is the ratio of amino acids (no. identified in peptides/no. in theoretical peptides from sequence data) expressed as a percentage.

[e] Alterations of expression were also detected by microarray analysis.

Marrakchi et al. (28) proposed that, in *S. pneumoniae*, FabR might synchronously regulate its own expression in addition to that of *fabM* and *fabK*, since a putative operator for *fabR* was identified upstream of each of the three genes. Our transcriptome data also suggest that *fabK*, *fabD*, *fabG*, *fabF*, *accB*, *fabZ*, *accC*, *accD*, and *accA* are coordinately regulated, because the changes in expression of these genes follow the same pattern after the induction of YycF. Likewise, the transcription of *fabR*, *fabH*, and *acpP* might be coregulated, as they show comparable decreases in transcription after YycF induction. In contrast, *fabM*, the first gene within the fatty acid biosynthetic gene cluster, was not significantly affected after YycF induction (Fig. 2C) and thus might be independently regulated. The two hexanucleotide direct repeats (Fig. 2B), deviating in only one nucleotide from the consensus sequence (5′-TGTWAHN$_5$TG TWAH-3′), of the YycF operators of *B. subtilis* and *S. aureus* (13, 24) were only identified upstream of *fabR*. In addition, an inverted repeat binding motif of the winged-helix motif of the MarR regulator (2) was identified adjacent to the −10 region of the putative promoter of fatty acid elongation genes. Thus, it is possible that both YycF and FabR, acting as a transcriptional repressor, may play a role in a regulatory cascade of the fatty acid biosynthetic genes.

In this study, we provided evidence from both proteomic and microarray transcription studies, as well as from an analysis of membrane fatty acid compositions, that in *S. pneumoniae* YycFG is involved in the regulation of the fatty acid biosynthesis pathways and in modulating the chain lengths of fatty acids.

TABLE 3. Fatty acid composition of total membrane lipid extracts from *S. pneumoniae* JNR/87(pLS1RGFP) and JNR7/87(pPL100)

Fatty acid(s) or ratio[a]	% of total fatty acid in indicated strain[b]	
	JNR7/87(pLS1RGFP)	JNR7/87(pPL100)
SFAs		
n-12:0	0.68 ± 0.26	0.19 ± 0.06
n-14:0	3.94 ± 0.45	2.28 ± 0.51
Anteiso-15:0	0.11 ± 0.01	0.12 ± 0.03
n-16:0	32.10 ± 1.76	29.83 ± 2.48
Iso-17:0	0.06 ± 0.04	0.28 ± 0.03
Anteiso-17:0	0.63 ± 0.07	0.59 ± 0.03
n-18:0	11.30 ± 1.80	15.06 ± 0.76
Σ SFAs	48.82 ± 4.39	48.35 ± 3.90
18:0/16:0	0.35 ± 0.04	0.50 ± 0.01
UFAs		
n-14:1	0.34 ± 0.10	0.09 ± 0.04
n-16:1Δ7	2.11 ± 0.46	0.76 ± 0.10
n-16:1Δ9	9.49 ± 1.99	3.35 ± 0.42
n-16:1Δ11	1.23 ± 0.30	0.94 ± 0.24
Σ 16:1	12.83 ± 2.75	5.05 ± 0.76
n-18:1Δ9	5.33 ± 0.72	5.11 ± 0.28
n-18:1Δ11	33.78 ± 1.76	41.04 ± 2.30
n-18:1Δ13	1.71 ± 0.25	2.77 ± 0.16
Σ 18:1	40.82 ± 2.73	48.92 ± 2.74
Σ UFAs	53.65 ± 5.48	53.97 ± 3.50
18:1/16:1	3.18 ± 0.49	9.68 ± 0.93

[a] SFA, saturated fatty acid; UFA, unsaturated fatty acid.

[b] Mean values and standard deviations of six independent experiments are depicted.

This finding is in agreement with a report by Martin et al. (29), who showed that a temperature-sensitive lethal mutant of *S. aureus* carrying a point mutation in *yycF* was more sensitive to macrolide and lincosamide antibiotics and unsaturated fatty acids. Macrolide susceptibility has also been shown to increase markedly in mutants involved in phospholipid biosynthesis, and it was proposed that YycF may have a role in the regulation of membrane composition. Alterations in the expression of fatty acid biosynthesis genes were not reported in a recent study by Ng et al. (32), in which changes in transcription patterns were characterized for *S. pneumoniae* cells depleted of *yycFG* expression. The most likely explanation for this lies in the fact that Ng et al. (32) only compiled a list of genes with changes of more than twofold in their relative transcript amounts, whereas we statistically analyzed all genes showing reproducible changes in transcript amounts of >1.75-fold in triplicate experiments. However, in common with the YycF depletion approach taken by Ng et al. (32), we found an increased expression of several genes involved in the stress response (e.g., *grpE*, *dnaK*, *hrcA*, and *groEL*) after long-term induction. Therefore, these transcriptional changes are presumably due to the physiological stresses provoked by the prolonged depletion or overexpression of YycF and not to direct control by the regulator. Most interestingly, the relative transcript levels of *lytB*, a cell wall hydrolase gene, and *pcsB* (a possible extracellular cell wall hydrolase gene) were both substantially increased in response to an increased expression of YycF (shown here) but were substantially decreased during YycF depletion (32), strongly suggesting that YycF plays a role in the regulation of these genes.

The relative transcript levels of other genes that might play a role in the biosynthesis or degradation of the cell envelope, such as *mreC* and two genes containing LysM domains (Sp0107 and Sp2063), were also affected by the induction of YycF expression. Interestingly, in *B. subtilis* YycF specifically activates the expression of *yocH*, which encodes a potential autolysin (24). The YycF protein in *B. subtilis* also seems to modulate the expression of *ftsAZ* (17, 24). However, in *S. pneumoniae* the *ftsAZ* cell division operon was not affected by the induction or repression of *yycF* expression (32; this work), suggesting that YycF might not regulate this operon as reported for *B. subtilis* (24).

The histidine kinase YycG contains a putative PAS/PAC domain near the cytoplasmic N terminus that has been shown in other proteins to be associated with responses to changes in redox potential, light, oxygen, or energy levels (18, 39, 41). Echenique and Trombe (14) suggested that the phosphorylated form of YycF in *S. pneumoniae* is involved in competence repression under microaerobiosis. Mutation of the PAS domain in YycG abolishes its kinase activity and allows the expression of competence under microaerobic conditions. Also, Kadioglu et al. (25) proposed that the YycFG TCS of the pneumococcus is involved in the adaptive response of the bacteria to changes in oxygen levels. Interestingly, changes in the fatty acid compositions of membrane lipid pools have been observed to occur in *S. aureus* cells transitioning between aerobic growth and anaerobic growth (43). Therefore, we suggest that this TCS regulates the membrane composition in response to oxygen and possibly other stimuli, such as redox potential and energy levels, via the PAS/PAC sensing domain in YycFG.

In addition, we have identified other potential members of the YycF regulon that are required for virulence, such as the iron transport system *pia* and several genes such as *lytB*, *pscB*, and Sp0107 that might play a role in cell wall biosynthesis and metabolism. In support of this hypothesis, a putative consensus DNA binding motif (5′-TGTHHHN$_2$TTGTHDH-3′) for YycF was found upstream of these genes as well as of *fabR* (Fig. 2C) and *yycF* in the StreptoPneumoList database (http://genolist.pasteur.fr).

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