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Characterization of a Novel Fucose-Regulated Promoter (P_{fcsK}) Suitable for Gene Essentiality and Antibacterial Mode-of-Action Studies in *Streptococcus pneumoniae*

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The promoter of the *Streptococcus pneumoniae* putative fuculose kinase gene (*fcsK*), the first gene of a novel fucose utilization operon, is induced by fucose and repressed by glucose or sucrose. When the streptococcal polypeptide deformylase (PDF) gene (*def1*, encoding PDF) was placed under the control of P_{fcsK}, fucose-dependent growth of the *S. pneumoniae* (P_{fcsK}::*def1*) strain was observed, confirming the essential nature of PDF in this organism. The mode of antibacterial action of actinonin, a known PDF inhibitor, was also confirmed with this strain. The endogenous fuculose kinase promoter is a tightly regulated, titratable promoter which will be useful for target validation and for confirmation of the mode of action of novel antibacterial drugs in *S. pneumoniae*.

Streptococcus pneumoniae is a widespread human pathogen and a major cause of community-acquired diseases such as pneumonia, otitis media, sinusitis, and meningitis (12). Established antibiotic treatments of pneumococcal infections have become less effective due to the emergence of drug-resistant isolates (34). A genomics-based strategy has been applied in the search for new drug targets to identify inhibitors active against this pathogen (25, 33). Once a lead antimicrobial compound has been discovered, it is fundamentally important to demonstrate that during chemical optimization the antibacterial activity continues to be related to inhibition of the specific target (25). Tightly regulated, titratable promoter systems that are able to modulate the levels of the protein target have proven to be invaluable tools for tracking the mechanism of antibacterial activity of novel inhibitors (2, 37). In addition, inducible promoters have been used in antimicrobial drug discovery for establishing gene essentiality and characterizing the function of essential drug targets (1, 9, 31). For *S. pneumoniae* only a limited number of regulated promoters have been studied. Heterologous promoter systems derived from nisin and tetracycline genes have been analyzed as tools for regulating gene expression, but their narrow titratable range and high basal levels of expression have compromised their use (1, 2, 7). The streptococcal promoter of the maltose operon has been characterized at the molecular level and shown to be inducible by maltose and repressible by sucrose in *S. pneumoniae* (23), but its use has been limited by its high basal expression levels. More recently, the raffinose operon has been identified and its promoter has been shown to be regulated, though its application for target validation or mode-of-action analysis has yet to be demonstrated (27). The expression of genes involved in sugar metabolism is known to be a regulated process in many bacterial species. With the availability of genomic sequence data for *S. pneumoniae*, it is now possible to identify and study many, and perhaps all, putative sugar metabolic genes and their associated promoter sequences.

Identification and bioinformatic analysis of the fucose gene cluster of *S. pneumoniae*. In an attempt to identify novel regulatable promoter systems in *S. pneumoniae*, DNA sequences from three pneumococcal genomes (strains 100993 [GlaxoSmithKline], R6 [15], and type 4 [32]) were examined for homology to known carbohydrate utilization operons of *Escherichia coli* and *Bacillus subtilis*. Fourteen putative carbohydrate utilization operons were identified, including those for cellobiose, fructose, fucose, galactose, glucose, lactose, maltose, mannitol, mannose, raffinose, sucrose, and trehalose (data not shown). Several of these operons contained homologs of genes involved in the regulation, uptake, and metabolism of sugars in bacteria. A putative fucose gene cluster containing 11 genes, some of which showed homology to the fucose catabolism genes of *E. coli* and *Haemophilus influenzae* (8, 19), was selected for further studies (Fig. 1A). The first gene of the putative operon encodes a protein exhibiting 37% identity to the rhamnulokinase of *Salmonella enterica* serovar Typhimurium (24) and 22% identity to fuculokinase of *H. influenzae* (10) and is consequently referred to as *fcsK*. The gene products encoded by the next two genes in the operon (*fcsA* and *fcsU*) showed 41 and 49% amino acid identity to *E. coli* FucA, a fuculose-1-phosphate aldolase involved in fucose catabolism (38) and *H. influenzae* FucU, a fucose operon pro-

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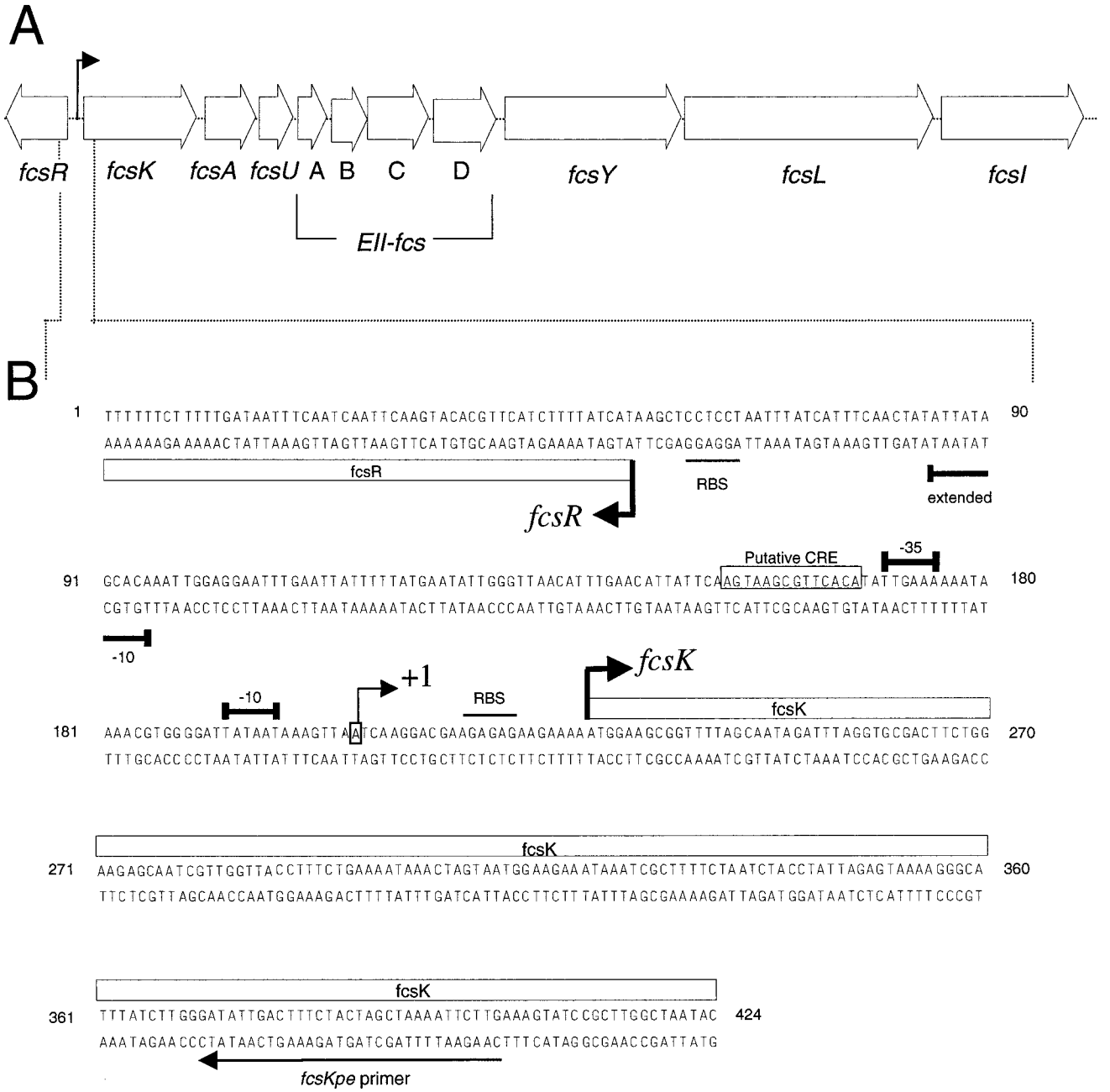

FIG. 1. Genetic organization of the *S. pneumoniae* putative fucose (*fcs*) gene cluster and DNA sequence of its promoter (P_{fcsK}). (A) The open reading frames and directions of transcription of the 11 genes in the putative fucose cluster are indicated: *fcsR* (fucose repressor protein), *fcsK* (fuculose kinase), *fcsA* (fuculose-1-phosphate aldolase), *fcsU* (fucose operon protein and putative oxidoreductase), EIIA-*fcs* to EIID-*fcs* (enzyme IIA to IID components of the phosphotransferase-phosphoenolpyruvate sugar uptake system), *fcsY* (hypothetical protein), *fcsL* (putative fucose-lectin binding protein), and *fcsI* (L-fucose isomerase). (B) DNA sequence of the intergenic *fcsR-fcsK* region. The translational start sites of the *fcsR* and *fcsK* genes, which are divergently transcribed, are indicated by bold arrows. The open reading frames have been boxed. Putative ribosome binding sites (RBS) and extended −10 and −10 and −35 promoter sequences are highlighted. The 5′-fluorescently tagged primer fcsKpe, used to determine the 5′ end of the *fcsK* transcript and hence the putative location of the P_{fcsK} promoter, is shown. For the primer extension reaction, 15 μg of DNase-treated RNA (isolated from *S. pneumoniae* R6 cells grown in a semidefined medium in the presence of 0.2% glucose and 1% fucose) was denatured together with 5 pmol of fcsKpe primer and reverse transcribed to cDNA. Samples were separated with a Perkin-Elmer ABI 377XL sequencer electrophoresis set, and the size of the primer extension product (196 ± 2 nucleotides) was determined with Perkin-Elmer ABI Prism Genescan Analysis 2.1 software. The transcriptional initiation site (+1) upstream of the *fcsK* gene, predicted by primer extension analysis, is indicated. A palindromic sequence showing similarity to the catabolite repression element (CRE) sequence of *B. subtilis* (16), determined with the MAST software program, is boxed.

tein (10) distantly related to proteins with oxidoreductase function, respectively. Downstream in the same orientation are four genes (EIIA-*fcs*, EIIB-*fcs*, EIIC-*fcs*, and EIID-*fcs*) whose products show 34, 35, 28, and 35% homology to mannose- or fructose-specific enzyme II components A, B, C, and D, respectively, of the phosphotransferase phosphoenolpyruvate sugar transport system (26). Two additional genes, designated *fcsY* and *fcsL* in this study, encode hypothetical proteins of unknown function (Fig. 1A), although FcsL contains a region of strong similarity to fucose-lectin binding proteins from *Anguilla japonica* (14). Distal to *fcsL* is another putative fucose metabolism gene, *fcsI*, whose product shows 62% identity to *H. influenzae* FucI, encoding a L-fucose isomerase that has been implicated in fucose catabolism (10). Divergently transcribed to the *fcsK* operon is a putative regulatory gene encoding FcsR (Fig. 1A), which shows 35% identity to LacR, the *Streptococcus mutans* lactose repressor (28), suggesting that the fucose operon is subject to negative regulation. DNA sequence analysis revealed putative promoters in the fucose gene cluster located upstream of both *fcsK* and *fcsR* (Fig. 1B). The genome organization of the fucose operon is conserved in all three publicly available *S. pneumoniae* genome sequences (6, 15, 32). However, the role of fucose in pneumococcal metabolism is unclear, since all the *S. pneumoniae* strains tested were unable to grow either in L- or D-fucose as the sole carbon source in a semidefined medium.

Transcriptional analysis of P_{fcsK} by primer extension. The transcriptional start site of P_{fcsK} was mapped by primer extension to an adenine residue 24 bp upstream of the *fcsK* initiation codon (Fig. 1B). Transcription is predicted to start 8 bp downstream of a canonical −10 RNA polymerase binding sequence (TATAAT) which is itself separated by 17 bp from a near-consensus sequence (TTGAAA) for the −35 region (Fig. 1B). A consensus extended −10 promoter (TGTGCTATAAT), which is common in *S. pneumoniae* (29), was identified upstream of the fucose repressor gene (*fcsR*), transcribed divergently to *fcsK*. A palindromic sequence (AGTAAGCGTTCA CA) possessing close identity (13 out of 14 bp) to the consensus sequence of the *B. subtilis* carbon catabolite repression element (TGt/aNANCGNTNa/tCA) was also located in the intergenic region between *fcsK* and *fcsR* (Fig. 1B) (16), suggesting that the fucose gene cluster is subject to catabolite repression. The exact role of FcsR and possibly CcpA (carbon catabolite binding protein) (16) in regulating the expression of this operon remains to be determined.

RT-PCR analysis of induction of the fuculose kinase gene (*fcsK*). Regulation of the presumptive promoter (P_{fcsK}) immediately upstream of *fcsK* was examined by growing wild-type *S. pneumoniae* R6 under 10 different growth conditions in a semidefined medium (AGCH [a basal medium containing casein hydrolysate, amino acids, vitamins, salts, albumin, and catalase] containing 0.2% [wt/vol] yeast extract [YE] [17]) supplemented with different sugars as carbon source at the time of inoculation. In the presence of glucose, sucrose, lactose, trehalose, fructose, or mannose, added at 1% (wt/vol) concentrations, *S. pneumoniae* grew to late logarithmic phase (optical density at 650 nm [OD_{650}] = 0.6) at approximately the same growth rates (data not shown). Since *S. pneumoniae* was unable to utilize 1% fucose or galactose for growth and showed a significantly reduced growth rate in the presence of 0.5% raffinose, 0.2% (wt/vol) glucose was added to the medium to support growth in these three cases (27). RNA was prepared from bacteria grown to late logarithmic phase (OD_{650} = 0.6), and the effect of the sugars on the levels of the *fcsK* transcript was quantified by reverse transcription-PCR (RT-PCR) analysis. Steady-state *fcsK* mRNA levels under the different growth conditions were compared to those found when the strain was grown in the presence of 0.2% (wt/vol) glucose alone (Fig. 2). Growth in glucose, sucrose, lactose, trehalose, fructose, and mannose had no significant effect on the levels of *fcsK* transcript (Fig. 2). Transcription of *fcsK* was induced by fucose and also by the structurally related sugar galactose. Levels of steady-state *fcsK* mRNA increased 23-fold in the presence of 1% L-fucose and 15-fold following the addition of 1% L-galactose (Fig. 2). Our analysis also indicated that levels of *fcsK* mRNA are very low in the presence of glucose or sucrose. Although *S. pneumoniae* does not grow on fucose, the promoter P_{fcsK} is functional and clearly induced by that sugar.

Regulation of P_{fcsK}::*luxAB*. Regulation of the fuculose kinase promoter in *S. pneumoniae* was further characterized by integrating a P_{fcsK}::*luxAB* transcriptional reporter system into the chromosome (Fig. 3A). Since *S. pneumoniae* is unable to grow on fucose as the sole carbon source, a second sugar substrate (sucrose or glucose) was added to support growth. To analyze regulation of P_{fcsK}, the *S. pneumoniae* (P_{fcsK}::*luxAB*) reporter strain was grown in media containing 0.3% sucrose and different concentrations of L-fucose added as the inducer at the start of growth (Fig. 3B). There was no difference in the growth rate of the *S. pneumoniae* reporter under the various conditions, with all cultures reaching a maximum OD_{650} of approximately 0.9 after 8 h (data not shown). Optimal P_{fcsK}::*luxAB* expression, as measured by luciferase activity, was observed during late logarithmic phase (OD_{650} = 0.6). The promoter fusion activity was shown to be titratable by varying the concentrations of fucose from 0.01 to 1% in 0.3% sucrose (Fig. 3B). Maximum induction was observed in medium containing >0.4% fucose as the inducing sugar in the presence of 0.3% sucrose (Fig. 3B). Relative to growth carried out in medium lacking fucose, there was an approximately 300-fold increase in luciferase activity following the addition of 0.4% fucose (Fig. 3B). Moreover, repression of P_{fcsK} by about 25-fold was observed in the presence of increasing concentrations of sucrose (Fig. 3B). A similar titratable effect was demonstrated when glucose was used to replace sucrose as the carbon source (data not shown). Clearly, P_{fcsK} can be very effectively repressed by sucrose or glucose. Under these test conditions, in which maximum fucose induction is measured in the presence of sucrose (a repressor of P_{fcsK}), the promoter shows a full dynamic range of approximately 7,500-fold. The measurement of the steady-state transcript levels of *luxAB* and *fcsK* by real-time quantitative PCR analysis, from the same reporter strain, following induction with fucose, showed dynamic ranges that correlated with luciferase activity (results not shown). For *S. pneumoniae*, sucrose-mediated repression of other sugar metabolic pathways has also been reported, though the exact mechanism(s) of this regulation is unknown (18, 23, 27). Transcription analysis with both RT-PCR and reporter gene fusion technology has identified fucose as the primary inducer of the P_{fcsK} promoter and indicated the extent of its regulation (Fig. 2 and 3).

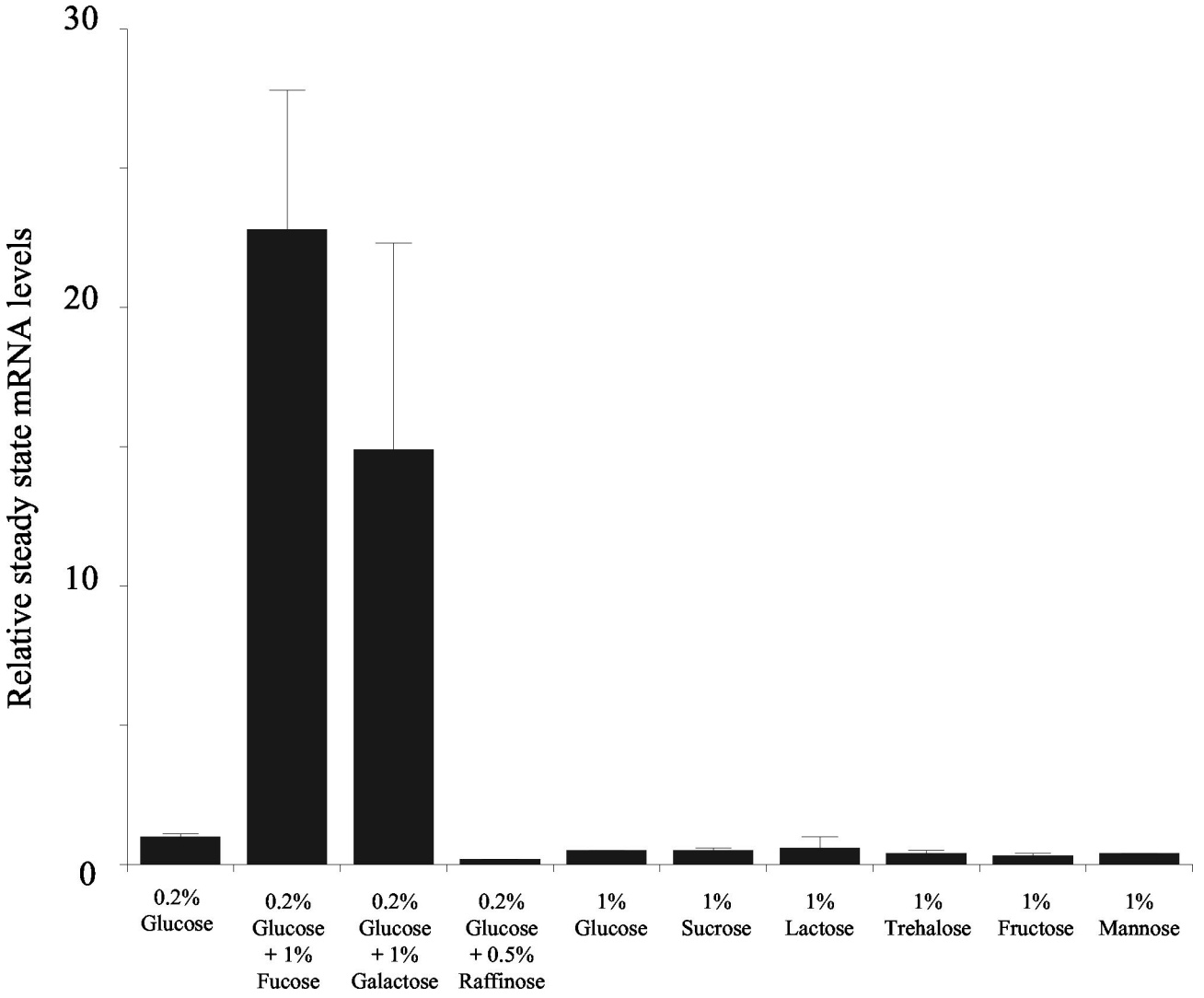

FIG. 2. Effects of different sugars on the induction of the fuculose kinase (*fcsK*) gene measured by quantitative, real-time SybrGreen RT-PCR. *S. pneumoniae* R6 was grown statically at 37°C in AGCH-YE medium and supplemented with different test sugars. Total RNA was extracted from late-logarithmic-phase-grown *S. pneumoniae* cells by using the Bio 101 FastRNA kit (Vista, Calif.) following glass bead cell disruption and a hot phenol lysis step (5). DNase-treated RNA was reverse transcribed to cDNA with a First Strand synthesis kit (Invitrogen). Relative levels of bacterial transcripts in each sample were quantified by PCR following SybrGreen dye incorporation (SybrGreen PCR core reagent kit; Applied Biosystems, Perkin-Elmer), and products were detected in real time with the 7700 sequence detection system (Applied Biosystems) as described previously (22, 35, 37). Template primers used in the PCRs are available on request. The quantity of cDNA estimated was normalized to a housekeeping gene, *era*. Changes in steady-state levels of *fcsK* mRNA in each sample were expressed relative to the uninduced control (0.2% glucose).

Utilization of P_{fcsK} for essentiality testing. The gene encoding polypeptide deformylase (PDF) has previously been shown to be essential in *E. coli* (11), *Staphylococcus aureus* (37), and *S. pneumoniae* (2, 20). To investigate the utility of the fucose-regulatable promoter for essentiality testing in *S. pneumoniae*, a promoter replacement strategy was used to place *def1* (encoding PDF) under the control of P_{fcsK} on the *S. pneumoniae* chromosome (Fig. 4A) (details of construction available on request). The resulting mutant strain, *S. pneumoniae* (P_{fcsK}::*def1*), was recovered in the presence of fucose. The mutant strain was tested for growth dependency on fucose by monitoring growth in 0.8% sucrose with various amounts of fucose as inducer (Fig. 4B). The higher concentration of sucrose in these experiments supports optimal growth of the control strain and maximizes the extent of repression of the P_{fcsK} promoter. In the presence of 0.1% fucose, *S. pneumoniae* (P_{fcsK}::*def1*) grew identically to the control wild-type strain *S. pneumoniae* R6 (data not shown). A decrease in the growth rate of the mutant strain was observed when the levels of fucose in the medium were reduced to 0.05%. Growth was not supported in 0.025% fucose, indicating that PDF levels had fallen below the threshold required for bacterial growth (Fig. 4B). The growth of the control strain *S. pneumoniae* R6 was not affected by the addition of fucose (results not shown).

To demonstrate that the fucose-dependent growth of *S. pneumoniae* (P_{fcsK}::*def1*) was due to a titration of PDF levels in

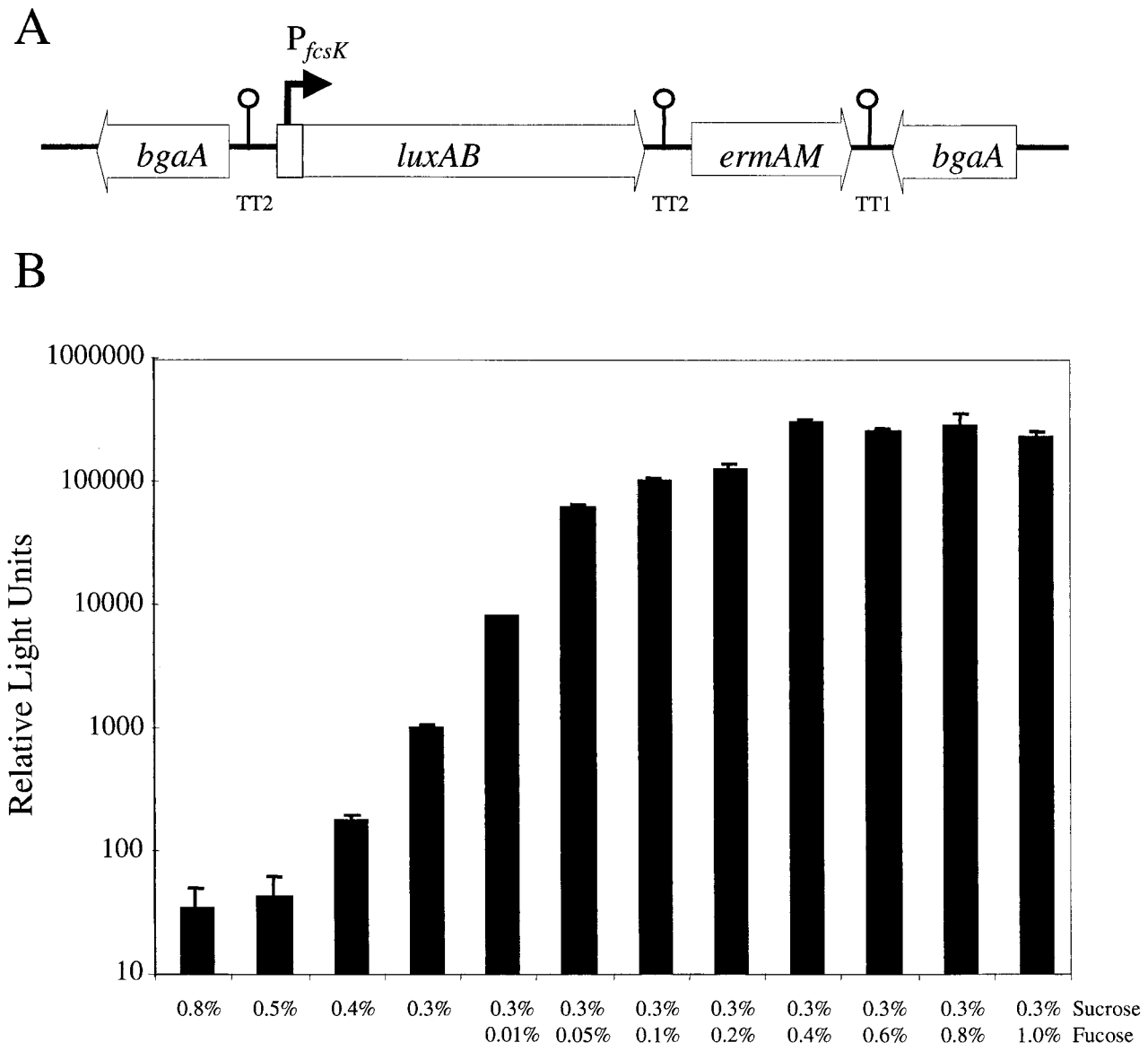

FIG. 3. Fucose induction and sucrose repression of P_{fcsK} in an *S. pneumoniae* (P_{fcsK}::*luxAB*) reporter strain. (A) Construction of a P_{fcsK}::*luxAB* transcriptional reporter fusion. A genetic map shows the organization of the P_{fcsK}::*luxAB* reporter cassette following integration into the chromosome of *S. pneumoniae* R6. Details of construction of the *luxAB* reporter strain in *S. pneumoniae* are available on request. Briefly, the cassette contains the promoter region of the fuculose kinase gene (P_{fcsK}) amplified from *S. pneumoniae* R6 and fused to a promoterless *luxAB* reporter gene from *Vibrio harveyi* (13). Transcriptional terminators of two large rRNA operons, TT1 and TT2, were amplified from *S. pneumoniae* R6 and introduced to flank the reporter fusion and prevent local transcriptional interference. The erythromycin resistance marker (*ermAM*) of *Enterococcus faecalis* was amplified from pAMβ1 for selection (21). The cassette was flanked by regions of the β-galactosidase (*bgaA*) structural gene (36). The construct was integrated into *S. pneumoniae* R6 at the *bgaA* locus by transformation (33). Erythromycin-resistant transformants were selected (5 μg of erythromycin/ml), and successful construction of the P_{fcsK}::*luxAB* reporter strain in single copy in the chromosome was confirmed by both diagnostic PCR and DNA sequencing. Arrows indicate the directions of transcription of the genes. Lollipop structures represent the transcriptional terminators. (B) Titration range of luciferase activity in the *S. pneumoniae* (P_{fcsK}::*luxAB*) reporter strain following growth in the presence of sucrose and fucose. To study the regulation of P_{fcsK} in *S. pneumoniae*, the *S. pneumoniae* (P_{fcsK}::*luxAB*) transcriptional reporter fusion strain was grown to late logarithmic phase (OD_{650} of about 0.6) in AGCH-YE medium containing different concentrations of sucrose and fucose. To measure luciferase activity, bacterial cells (250 μl) were transferred to a microtiter plate and 2 μl of *n*-decyl aldehyde substrate (Sigma) was added. Light output from the reaction was counted for 2 s with a MicroLumat LB96P luminometer (EG & G Berthold). The relative light units were calculated as the light output per OD_{650} unit per milliliter of culture (3, 13).

the cell, total cellular protein was prepared from *S. pneumoniae* R6 and P_{fcsK}::*def1* strains (Fig. 4C) and probed with antibodies raised against *S. pneumoniae* PDF. PDF protein levels were approximately fivefold lower in the regulatable strain (lanes 7 and 9, Fig. 4C) than in the wild-type strain (lanes 6 and 8, Fig. 4C), even when cells were grown under conditions that support optimal growth (0.8% sucrose and 0.1 or 0.8% fucose [Fig. 4B]). In these studies, the presence of sucrose

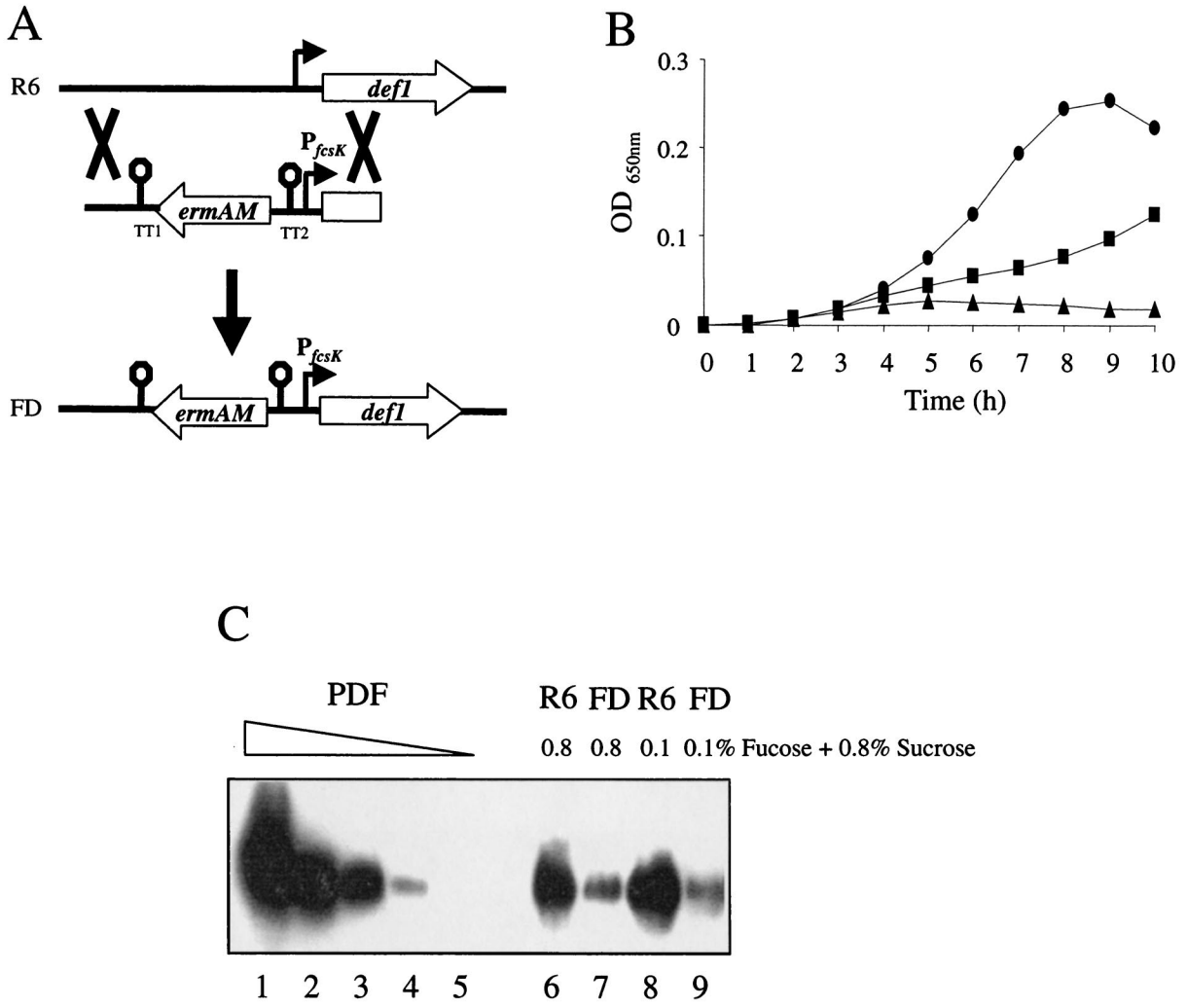

FIG. 4. Fucose-dependent growth of an *S. pneumoniae* (P_{fcsK}::*def1*) regulatable strain. (A) An *S. pneumoniae def1* regulatable strain (FD) was constructed by placing *def1* under the control of the P_{fcsK} inducible promoter in the chromosome of *S. pneumoniae*. Briefly, by using a three-piece PCR strategy of overlapping primers (33), a promoter replacement cassette was constructed containing the P_{fcsK} promoter, transcriptional terminators (TT1 and TT2) located 5′ of the P_{fcsK} promoter, and an independent erythromycin resistance marker (*ermAM*) (21) and flanked by DNA sequences of the gene immediately upstream of *def1* and the start of the *def1* open reading frame. The construct was integrated into *S. pneumoniae* R6 at the *def1* locus by transformation (33). An erythromycin-resistant mutant of P_{fcsK}::*def1* (FD) was recovered in the presence of added fucose, and both diagnostic PCR and DNA sequencing confirmed its chromosomal organization. (B) The effect of fucose on growth of an *S. pneumoniae* (P_{fcsK}::*def1*) regulatable strain. *S. pneumoniae* strains were grown statically at 37°C in AGCH-YE medium containing 0.8% (wt/vol) sucrose and L-fucose at 0.1% (●), 0.05% (■), or 0.025% (▲) (wt/vol). Growth experiments were performed in triplicate in a microtiter plate format with a SpectraMax250 spectrophotometer (Molecular Devices) as described previously (37). (C) Western blot analysis of PDF levels in *S. pneumoniae* (P_{fcsK}::*def1*) FD and R6. *S. pneumoniae* R6 (lanes 6 and 8) and *S. pneumoniae* (P_{fcsK}::*def1*) FD (lanes 7 and 9) were grown in AGCH-YE medium plus 0.8% (wt/vol) sucrose and fucose at 0.8% (lanes 6 and 7) or 0.1% (lanes 8 and 9) (wt/vol) final concentrations. Mid-logarithmic-phase cultures (OD_{650} of approximately 0.15) were resuspended in sterile distilled water to an equivalent of an OD_{650} of 4 (path length, 1 cm), and total cell lysates were prepared as described previously (30). Ten microliters of total protein samples (lanes 6 to 9) and *S. pneumoniae* PDF protein standards in 50-, 10-, 2-, 0.4-, and 0.08-ng amounts (lanes 1 to 5, respectively) were loaded onto each lane of a 10% NuPAGE Bis-Tris resolving gel and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (37). Western blotting was performed as described previously (37), and protein samples were probed with rabbit polyclonal antiserum raised against *S. pneumoniae* PDF (Covance Research Products) (diluted 1/1,000) and anti-rabbit horseradish peroxidase (Sigma) as secondary antibody (diluted 1/10,000).

serves both as the carbon source for growth and as the repressor of P_{fcsK}. Sucrose-mediated catabolite repression of P_{fcsK}::*def1* may suppress fucose induction, explaining why, even at conditions of maximum induction, the *fcsK* promoter cannot reach the expression level of the natural *def1* promoter. Since there was no difference in growth between wild-type and mutant strains, 20% of wild-type levels of PDF is sufficient (i.e., not rate limiting) for growth under these conditions.

Bioinformatic analysis of the *S. pneumoniae* genome organization showed that *def1* is the first gene in a two-gene operon, which also includes *yacO* (encoding a putative RNA methyltransferase). Downregulation of *yacO* is not responsible

for the growth defect observed in *S. pneumoniae* (P_{fcsK}::*def1*) at low fucose concentrations because allelic replacement of this gene has shown that it is not essential for in vitro growth in *S. pneumoniae* (data not shown). The clear dose dependency on fucose for growth of the *def1*-regulatable strain demonstrates that *def1* is indispensable for cell viability in *S. pneumoniae*.

Application of established *S. pneumoniae* regulatable systems for the purpose of gene essentiality testing has been limited by relatively high levels of basal expression. For example, regulation of expression of *def1* in *S. pneumoniae* has previously been achieved with the use of a tetracycline promoter system (2); however, no change in cell growth rate was observed in the absence of inducer, indicating that *def1* expression cannot be downregulated to the level required for confirmation of essentiality. In contrast, the essential nature of PDF in *S. pneumoniae* could be demonstrated with the use of P_{fcsK}.

Clearly, induction by fucose can overcome repression by sucrose, but the full level of induction is not achieved, and as a consequence, strains in which a gene is under the control of P_{fcsK} generally underproduce the target protein. Given that protein levels can be titrated down with decreasing amounts of inducer, gene essentiality can still be demonstrated.

Application of *S. pneumoniae* (P_{fcsK}::*def1*) for antibiotic mode-of-action studies. *S. pneumoniae* (P_{fcsK}::*def1*) produces five times less PDF than does the wild-type strain (Fig. 4C) and should therefore be hypersensitive to any PDF inhibitor. To demonstrate the utility of this strain for studying antibiotic mode of action, the MICs of a number of antibiotics were determined for *S. pneumoniae* (P_{fcsK}::*def1*) and R6 following static overnight growth at 37°C in AGCH-YE medium containing 0.8% sucrose and 0.8% fucose. Indeed, the underexpressing *def1* strain (MIC = 0.125 to 0.25 μg/ml) showed a 32- to 64-fold increase in sensitivity to actinonin, a potent inhibitor of PDF activity (4), with respect to the wild-type strain (MIC = 8 to 16 μg/ml). In contrast, the sensitivity of the strain to a number of known inhibitors of DNA replication, transcription, translation, cell wall biosynthesis, and fatty acid biosynthesis remained unchanged. These results are consistent with inhibition of PDF as the reason for the antibacterial activity of actinonin and demonstrate the utility of such strains in mode-of-action studies for potential antibacterial agents in *S. pneumoniae*.

Strains in which expression of the target protein is under regulation are very powerful tools in antibacterial mode-of-action studies because underexpression of the essential protein should lead to a concomitant increase in sensitivity to specific inhibitors. This study is the first case reported in the literature of an endogenous *S. pneumoniae* promoter whose inducible-repressible characteristics allow its utilization for both essentiality and mode-of-action studies.

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