JOURNAL OF BACTERIOLOGY, Oct. 2011, p. 5207–5215 Vol. 193, No. 19
0021-9193/11/$12.00 doi:10.1128/JB.05170-11

Peptide-Regulated Gene Depletion System Developed for Use in *Streptococcus pneumoniae*[▽]

Kari Helene Berg, Truls Johan Biørnstad, Daniel Straume, and Leiv Sigve Håvarstein*

Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, N-1432 Ås, Norway

Received 27 April 2011/Accepted 20 July 2011

To facilitate the study of pneumococcal genes that are essential for viability or normal cell growth, we sought to develop a tightly regulated, titratable gene depletion system that interferes minimally with normal cellular functions. A possible candidate for such a system is the recently discovered signal transduction pathway regulating competence for natural transformation in *Streptococcus thermophilus*. This pathway, which is unrelated to the ComCDE pathway used for competence regulation in *Streptococcus pneumoniae*, has not been fully elucidated, but it is known to include a short unmodified signaling peptide, ComS*, an oligopeptide transport system, Ami, and a transcriptional activator, ComR. The transcriptional activator is thought to bind to an inverted repeat sequence termed the ECom box. We introduced the ComR protein and the ECom box into the genome of *S. pneumoniae* R6 and demonstrated that addition of synthetic ComS* peptide induced the transcription of a luciferase gene inserted downstream of the ECom box. To determine whether the ComRS system could be used for gene depletion studies, the *licD1* gene was inserted behind the chromosomally located ECom box promoter by using the Janus cassette. Then, the native versions of *licD1* and *licD2* were deleted, and the resulting mutant was recovered in the presence of ComS*. Cultivation of the *licD1 licD2* double mutant in the absence of ComS* gradually affected its ability to grow and propagate, demonstrating that the ComRS system functions as intended. In the present study, the ComRS system was developed for use in *S. pneumoniae*. In principle, however, it should work equally well in many other Gram-positive species.

Gene disruption studies have shown that the genome of *Streptococcus pneumoniae* R6 contains at least 133 essential genes, 32 of which have no known function (23, 25). As these studies were carried out with laboratory-grown pneumococci, it is reasonable to assume that additional genes are essential for survival under natural conditions. For obvious reasons, functional studies of essential genes are experimentally demanding. The best approach is probably to express essential genes ectopically under the control of a tightly regulated, titratable promoter. This allows deletion of the native gene, while the level of transcription of the ectopically expressed gene can be manipulated to gain insight into its function. The same technique ought to be applied to studies of growth-defective genes whose absence affects bacterial growth and proliferation. In this way it should be possible to avoid the selection pressure exerted by deletion of growth-defective genes that gives rise to suppressor mutations which mask or distort the real phenotype of the mutant.

Ideally, gene expression/depletion systems should not interfere with the normal physiology of the host bacterium. The ComRS signal transduction pathway, which regulates competence for natural transformation in *Streptococcus thermophilus* (9), has no close homologs in *S. pneumoniae.* We therefore considered it a promising tool for gene depletion studies of the pneumococcus. The *licD1* and *licD2* genes, which are involved in the synthesis of teichoic acid in *S. pneumoniae* (26), were selected as target genes in an initial test of the system. The *licD* genes are located on a transcriptional unit consisting of *tacF*, *licD1*, and *licD2*. The first gene in the operon, *tacF*, encodes a transporter that is required for the transport of teichoic acid subunits (or polymerized chains) across the cytoplasmic membrane (1, 5). In *S. pneumoniae*, the chemical structure of the repeating units in cell wall teichoic acid (WTA) and lipoteichoic acid (LTA) are identical. In general, each repeat unit contains two phosphoryl choline residues, one attached to the central *N*-acetyl-D-galactosamine residue and the other to the ribitol-linked *N*-acetyl-D-galactosamine residue (8). LicD1 and LicD2 attach phosphoryl choline residues to the teichoic acid subunits on the cytoplasmic side of the membrane, before they are exported by TacF (5). As TacF has a strict specificity for subunits containing phosphoryl choline, extracellular synthesis of WTA and LTA will probably be arrested in the absence of LicD1 and LicD2. It has been reported that the *licD2* gene of *S. pneumoniae* can be readily deleted, while attempts to construct a *licD1* deletion mutant were unsuccessful (26). This result suggests that *licD1* might be an essential gene. Alternatively, the observed lethal phenotype of the Δ*licD1* mutant could be due to a polar effect on the downstream *licD2* gene which is exerted by the pIH1 plasmid used to disrupt the *licD1* gene by insertion-duplication mutagenesis. Zhang and coworkers (26) favored the latter alternative and predicted that deletion of both *licD* genes is lethal for *S. pneumoniae.* Our results confirmed this prediction but showed that it is possible to construct a pneumococcal *licD1 licD2* double deletion mutant if *licD1* is ectopically expressed by the ComRS system. Removal of ComS* from the growth medium had no immediate effect, but after 4 to 6 h severe morphological abnormalities were observed. A few hours later the stress imposed by the gradual reduction in LicD1 expression culminated in growth

* Corresponding author. Mailing address: Department of Chemistry, Biotechnology, and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway. Phone: 47-64965883. Fax: 47-64965901. E-mail: sigve.havarstein@umb.no.

▽ Published ahead of print on 29 July 2011.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype/relevant features[a]	Reference/source
S. pneumoniae strains		
R704	R6 derivative, *comA*::*ermAM*; Ery^r	J. P. Claverys[c]
RH1	R704, but *ebg*::*spc*; Ery^r Spc^r	13
RH426	RH425, but ΔIS*1167*::Janus[b]; Ery^r Kan^r	14
SPH124	RH426, but with replacement of Janus cassette by P_{comR}::*comR*; Ery^r Sm^r	This study
SPH125	SPH124, but with Janus cassette inserted between *cpsO* and *cpsN*; Ery^r Kan^r	This study
SPH126	SPH125, but with replacement of Janus cassette by P_{comX}::*luc*; Ery^r Sm^r	This study
SPH127	SPH126, but with replacement of spr0324 by Janus cassette; Ery^r Kan^r	This study
SPH128	SPH127, but with replacement of Janus cassette by *amiA3*; Ery^r Sm^r	This study
SPH129	SPH126, but with replacement of *comR* by Janus cassette; Ery^r Kan^r	This study
SPH130	SPH129, but with replacement of Janus cassette with P_1::P_{comR}::*comR*; Ery^r Sm^r	This study
SPH131	SPH130, but with replacement of *luc* by Janus cassette; Ery^r Kan^r	This study
SPH132	SPH131, but with replacement of Janus cassette by *licD1*; Ery^r Sm^r	This study
SPH135	SPH132, but with replacement of native *licD1* and *licD2* genes by Janus cassette; Ery^r Kan^r	This study
SPH136	SPH130, but with replacement of *luc* by the *aacA-aphD* kanamycin resistance gene; Ery^r Sm^r Kan^r	This study
Plasmids		
pR424	ColE1 (pEVP3) derivative; Cm^r; carries the *luc* gene	4
pFW13	Carries the *aacA-aphD* kanamycin resistance gene	22

[a] Cm, chloramphenicol; Ery, erythromycin; Kan, kanamycin; Sm, streptomycin.
[b] Janus indicates the presence of a *kan*::*rpsL*$^+$ cassette (24).
[c] Gift from Jean-Pierre Claverys.

arrest followed by autolysis. We believe the ComRS system will become a valuable addition to the genetic toolbox available for *S. pneumoniae*.

MATERIALS AND METHODS

Construction of *S. pneumoniae* mutants. *S. pneumoniae* strains and plasmids used in this work are described in Table 1. All transformations and experiments were carried out in C medium (18) at 37°C. However, Todd-Hewitt (Difco) agar plates containing the appropriate antibiotic were used for selection of transformants. The sequences of all primers used are given in Table 2. To construct mutant strains, DNA was introduced into the recipients by natural transformation. Bacterial cultures were grown to an optical density at 550 nm (OD_{550}) of ~0.05 to 0.1 and induced to competence by adding synthetic competence-stimulating peptide (CSP) (11) to a final concentration of 250 ng ml^{-1}. Then, the transforming DNA was added, and the cultures were further incubated for 120 min at 37°C. Selection of transformed cells was carried out on Todd-Hewitt agar containing the appropriate antibiotic at the following concentrations: streptomycin (Sm), 200 μg ml^{-1}; kanamycin (Kan), 400 μg ml^{-1}. When needed, 2 μM synthetic ComS* (NH_2-LPYFAGCL-COOH) (Genosphere Biotechnologies) was included in the C medium during growth and transformation as well as in the Todd-Hewitt agar plates.

Pneumococcus strain SPH124 was derived from strain RH426 by replacing its Janus cassette (24) with the *stu0270* gene (designated *comR*) from *S. thermophilus* by double-crossover homologous recombination. Janus is a *kan rpsL*$^+$ DNA cassette that confers resistance to kanamycin and dominant sensitivity to streptomycin in a streptomycin-resistant background. Replacement of the Janus cassette restores streptomycin resistance and kanamycin sensitivity. Fragments corresponding to the ~1,000-bp upstream and downstream regions of the Janus cassette in RH426 were amplified using the primer pairs AmiF/AmiR and TreF/TreR, respectively. *comR*, including its upstream promoter region, was amplified using the primer pair khb43/khb29 with genomic DNA from *S. thermophilus* LMG18311 (2) as template and then fused to the upstream and downstream fragments by overlap extension PCR using the primers AmiF and TreR according to the method of Higuchi et al. (12). All PCR-based DNA-fragment fusions described in the present work were carried out using the method of Higuchi and coworkers.

Strain SPH125 was constructed by inserting the Janus cassette between the capsular genes *cpsO* (spr0323) and *cpsN* (spr0322) of the SPH124 strain. The Janus cassette was amplified by PCR using the primer pair khb41/khb42 with genomic DNA from RH426 as template. An ~800-bp DNA fragment corresponding to the 5′ end of the *cpsO* gene was amplified using the primers khb31 and khb32, whereas an ~800-bp DNA fragment corresponding to the 3′ region of the *cpsN* gene was amplified using the primers khb33 and khb34. These two fragments were subsequently fused to the 5′ and 3′ ends of the Janus cassette, respectively, using the primers khb31 and khb34.

Next, the Janus cassette in SPH125 was replaced by P_{comX}::*luc*, giving rise to strain SPH126. First, the promoter P_{comX} was amplified from *S. thermophilus* LMG18311 genomic DNA using the primers khb35 and khb36. The *luc* gene was amplified from pR424 (4) using the primer pair khb37/khb38. P_{comX} was then fused to the 5′ end of *luc* using the primers khb35 and khb38. The resulting fragment was subsequently fused to the *cpsO* upstream and *cpsN* downstream fragments described above, using the primers khb31 and khb34.

To explore a possible effect of expression of the oligopeptide-binding protein AmiA3, the *amiA3* (*stu1445*) gene from *S. thermophilus* was inserted into the capsular locus of SPH126, replacing the spr0324 gene. spr0324 was first replaced by a Janus cassette, giving rise to SPH127. The Janus cassette was amplified from RH426 using the primer pair khb54/khb55. Fragments corresponding to the ~1,000-bp upstream and downstream regions of *stu0324* were amplified using the primer pair khb50/khb51 and khb52/khb53, respectively. The two fragments were fused to the Janus cassette using the primers khb50/khb53. Next, *amiA3* and its promoter were amplified from *S. thermophilus* LMG18311 using the primer pair khb58/khb59. The resulting fragment was fused to the upstream and downstream flanking fragments described above, using the primers khb50 and khb53. SPH127 was transformed with the resulting DNA fragment, resulting in strain SPH128.

To construct the strain SPH130, which expresses *comR* constitutively from the synthetic promoter P_1 (14), *comR* in SPH126 was first replaced with the Janus cassette, giving rise to SPH129. The fragment used was amplified from RH426 using the primer pair AmiF/TreR. The Janus cassette was then replaced by a fragment consisting of *comR*, its native promoter P_{comR}, and the synthetic P_1 promoter (P_1::P_{comR}::*comR*), resulting in SPH130. The P_1::P_{comR}::*comR* fragment was constructed as follows: The *S. thermophilus comR* gene, including its promoter, was amplified with the primers khb72 and khb29, giving rise to a fragment with the P_1 promoter added at its 5′ end. Fragments corresponding to the ~1,000-bp upstream and downstream regions of the Janus cassette in SPH129 were amplified using the primer pairs AmiF/AmiR1 and TreF/TreR, respectively. The two fragments were then fused to the P_1::P_{comR}::*comR* fragment by using the primers AmiF and TreR.

To express *licD1* (spr1151) ectopically from the P_{comX} promoter, the *luc* gene was first replaced by a Janus cassette in SPH130, giving rise to SPH131. The Janus cassette was amplified from RH426 using the primers khb60 and khb42. The primers khb31 and khb36 were then used to amplify an ~950-bp DNA fragment corresponding to the *cpsO*::P_{comX} region flanking the 5′end of *luc*, whereas the primers khb33 and khb34 were used to amplify an ~800-bp DNA fragment corresponding to the *cpsN* region flanking the 3′ end of *luc*. The two fragments were then fused to the 5′ and 3′ ends of the Janus cassette, respectively, using the primers khb31 and khb34. SPH132 was constructed by trans-

TABLE 2. Primers

Primer	Oligonucleotide sequence (5′→3′)	Reference
16S F	ACCGCATAAGAGTGGATG	20
16S R	CAACGCAGGTCCATCTGGTA	20
AmiF	CGGTGAAGGAAGTAAGAAGTTT	14
AmiR	TCAAACTTATCAAGCGCAATGC	This study
AmiR1	AATGGAACCTCCACAAGTATTTTCTAGTATTATAGCACATTTAATCAAACTTATCAAGCGCAATGCCTTTG	14
cbpDF	ATGAAAATTTTACCGTTTATAG	This study
cbpDR	ATTTCCTCTGGAATAGGCATAG	This study
Kan484.F	GTTTGATTTTTAATGGATAATGTG	13
khb29	GGGTAAATCCCTTATAGATATTATGGAGTTTCTATAAACCAT CTGCCAATT	This study
khb31	ATAACAAATCCAGTAGCTTTGG	This study
khb32	GGTCTAGAGATGATTTTAATTAC	This study
khb33	TTTCTAATATGTAACTCTTCCCAAT	This study
khb34	CATCGGAACCTATACTCTTTTAG	This study
khb35	ATTAAAATCATCTCTAGACCGGTTTTGACTCTATCTCGCTT	This study
khb36	TGAACCTCCAATAATAAATATAAAT	This study
khb37	ATTTATATTTATTATTGGAGGTTCAATGAGATCCGCCAAAAACATA	This study
khb38	ATTGGGAAGAGTTACATATTAGAAATTACAATTTGGGCTTTCCGCC	This study
khb41	ATTAAAATCATCTCTAGACCGTTTGATTTTTAATGGATAATGTG	This study
khb42	ATTGGGAAGAGTTACATATTAGAAACTTTCCTTATGCTTTTGGAC	This study
khb43	GCATTGCGCTTGATAAGTTTGAGGATAATCAAGATTTATCTTATAAA	This study
khb50	CCGATGCAGAAATGGTTGAG	This study
khb51	GATAATCGTACATCTGAAGCTC	This study
khb52	TTGGTAAACTACGAACCGCTAG	This study
khb53	TCATTGTAAGCGCCCAATAAC	This study
khb54	GAGCTTCAGATGTACGATTATCGTTTGATTTTTAATGGATAATGTG	This study
khb55	CTAGCGGTTCGTAGTTTACCAACTTTCCTTATGCTTTTGGAC	This study
khb58	GAGCTTCAGATGTACGATTATCAGGAATTACAGTTGTTATCGTG	This study
khb59	CTAGCGGTTCGTAGTTTACCAACGTGTCTTCATCACACTGAAC	This study
khb60	ATTTATATTTATTATTGGAGGTTCAGTTTGATTTTTAATGGATAATGTG	This study
khb61	ATTTATATTTATTATTGGAGGTTCAATGAAACAACTAACCGTTGAAG	This study
khb62	ATTGGGAAGAGTTACATATTAGAAACCTAATCCTCCAATTTATAAGC	This study
khb63	TTTAGATCGCCTCTTCCTCG	This study
khb64	TTTAAACTCCTATGATTTTTTA	This study
khb67	TAAAAAATCATAGGAGTTTAAAGTTTGATTTTTAATGGATAATGTG	This study
khb68	ATTTCTTTTTTTTCTAAATATTGCATCTTTCCTTATGCTTTTGGAC	This study
khb69	ATTTCTTTTTTTTCTAAATATTGCATTTTAAACTCCTATGATTTTTTA	This study
khb70	TAAAAAATCATAGGAGTTTAAAATGCAATATTTAGAAAAAAAAGAAAT	This study
khb72	TTAAATGTGCTATAATACTAGAAAATACTTGTGGAGGTTCCATTGGATAATCAAGATTTATCTTATAAA	This study
khb79	ATATTTATTATTGGAGGTTCAATGAATATAGTTGAAAATGAAATATG	This study
khb80	AGAGTTACATATTAGAAACTCCGGATGCATATGCATGC	This study
khb84	GTCCAAAAGCATAAGGAAAGCTATAAGTATGTGGCTCTTCG	This study
khb85	CTGCTGACGAAGATGGTGAG	This study
khb86	GTCGACAATGCTCAAGTCTGTTTTAAACTCCTATGATTTTTTA	This study
khb87	TAAAAAATCATAGGAGTTTAAAACAGACTTGAGCATTGTCGAC	This study
khb88	ATTAAAATCATCTCTAGACCAAAGCCGGGAATTTTCCCGGCTTTTTTCTTGGTTTTGACTCTATCTCGCTT	This study
khb89	AAGAAAAAAGCCGGGAAAATTCCCGGCTTTGGTCTAGAGATGATTTTAATTAC	This study
lucF	ACCAGAGTCCTTTGATCGTG	This study
lucR	GAAAATAGGGTTGGTACTAGC	This study
RpsL41.R	CTTTCCTTATGCTTTTGGAC	13
TreF	CTCCATAATATCTATAAGGGATTTA	14
TreR	GTGACGGCAGTCACATTCTC	14

forming SPH131 with a PCR fragment consisting of the *licD1* gene flanked by appropriate targeting regions. *licD1* was amplified from *S. pneumoniae* RH1 using the primers khb61and khb62 and fused to the same flanking regions as described for the Janus cassette.

Strain SPH135 was constructed by replacing both native *licD* genes (*licD1* [spr1151] and *licD2* [spr1152]) in SPH132 with the Janus cassette. The Janus cassette was amplified by PCR using the primer pair khb67/RpsL41.R with genomic DNA from RH426 as template. The primers khb63 and khb64 were used to amplify an ~800-bp DNA fragment corresponding to the region flanking the 5′end of the native *licD1* gene, whereas the primers khb84 and khb85 were used to amplify an ~800-bp DNA fragment corresponding to the region flanking the 3′ end of *licD2*. The two fragments were then fused to the 5′ and 3′ ends of the Janus cassette, respectively, using the primers khb63 and khb85. When SPH132 was transformed with the resulting PCR product, 2 μM ComS was supplied to the growth medium and the agar plates used for selection of kanamycin-resistant transformants to ensure that *licD1* was expressed ectopically from the P_{comX} promoter.

Strain SPH136 was derived from SPH130 by replacing *luc* with a kanamycin resistance gene (*aacA-aphD*), so that kanamycin resistance was induced by ComS induction. *aacA-aphD* was amplified from pFW13 (22) using the primers khb79 and khb80. The resulting DNA fragment was fused to the *cpsO* upstream and *cpsN* downstream fragments described above, by using the primers khb31 and khb34.

Luciferase reporter assays. Mutants harboring the *luc* gene fused to P_{comX} were grown in C medium to an OD_{492} of ~0.3. Cultures of the different mutants were then diluted to an OD_{492} of ~0.05, from which 280 μl of each diluted culture was mixed with 20 μl D-luciferin (10 mM) from *Photinus pyralis* (Thermo Scientific) in a 96-well Corning NBS clear-bottom plate. The plate was incubated in a Fluostar Optima luminometer (BMG Labtech) at 37°C. The OD_{492} and luminescence were measured automatically every 10 min throughout the exper-

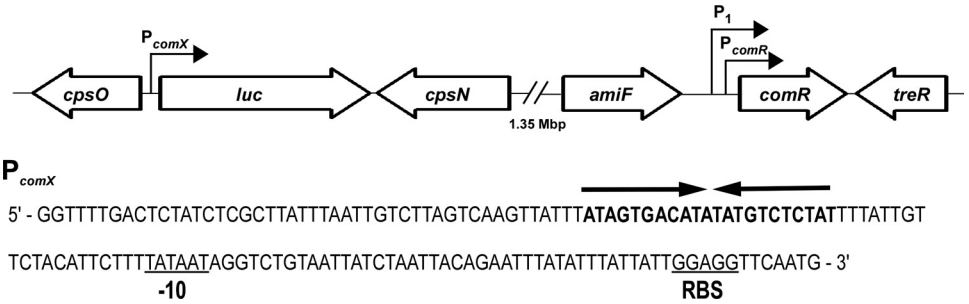

FIG. 1. Schematic diagram depicting two genetic regions of *S. pneumoniae* mutant SPH130 containing the inserted P_{comX}::*luc* and P_1::P_{comR}::*comR* constructs. The promoters P_{comX}, P_{comR}, and P_1 are indicated. The sequence of P_{comX} is shown below the diagram, with the predicted ComR-binding site (ECom box) indicated by arrows (9). P_{comR} represents the native ComR promoter, while P_1 represents a synthetic constitutive promoter inserted upstream of P_{comR}. The Pribnow box and the ribosome-binding site (RBS) are underlined.

iment. When performing the ComS* titration experiments, the following concentrations of ComS* were added to cell cultures that had reached an OD_{492} of 0.1: 10 μM, 1.25 μM, 0.63 μM, 0.31 μM, 0.16 μM, 0.08 μM, or 0 μM.

Depletion assays. Bacterial cultures were grown to an OD_{492} of 0.3 in the presence of 2 μM ComS*, pelleted by centrifugation, and washed once with C medium to remove excess ComS*. The washed cells were resuspended in fresh C medium with (2 μM) or without ComS* to an OD_{492} of ~0.05 and 2-fold diluted in a 96-well NBS clear-bottom plate (Corning). The plate was incubated in a Fluostar Optima luminometer (BMG Labtech) at 37°C. Luciferin-luciferase luminescence and the OD_{492} were measured automatically by the luminometer at 10-min intervals.

Western analysis. Pneumococcal cells (SPH128) were grown to an OD_{550} of 0.1 before splitting the culture into eight parallels that were subjected to 10 μM, 2.5 μM, 0.63 μM, 0.16 μM, 0.04 μM, 0.01 μM, 0.0025 μM, or no ComS*. After 1 h at 37°C, 10-ml culture aliquots were harvested by centrifugation at 4°C at 4,000 × *g* for 10 min. The cells were washed once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.2) before their total protein contents were analyzed by SDS-PAGE as described by Laemmli (19). After gel electrophoresis, the separated proteins were electroblotted onto a polyvinylidene fluoride membrane (Bio-Rad) using a Trans-Blot SD semidry transfer cell from Bio-Rad. Following blocking using 5% (wt/vol) skim milk powder dissolved in Tris-buffered saline–Tween (TBST; 150 mM NaCl, 0.1% (vol/vol) Tween 20, Tris-HCl [pH 7.6]), the membrane was incubated overnight with the primary antibody (antiluciferase antibody produced in rabbit from Sigma) at 4°C. After washing the filter 3 times in TBST, it was incubated with a secondary antibody (polyclonal goat anti-rabbit immunoglobulin–alkaline phosphatase [Dako]) at room temperature for 1 h, followed by 3 washes in TBST. The primary antibody was diluted 1:6,000 in TBST containing 5% (wt/vol) skim milk powder and 15% (vol/vol) soluble protein extract from *S. pneumoniae* RH1 to reduce unspecific background signals. The extract was made by passing 20 ml of an RH1 culture (OD_{550}, 0.3) through a French press. The secondary antibody was diluted 1:2,000 in TBST containing 5% (wt/vol) skim milk powder. The immunoblots were developed using the 5-bromo-4-chloro-3-indolylphosphate–Nitro Blue Tetrazolium liquid substrate from Sigma as described by the manufacturer.

Microscopy. To examine the effect of reducing the level of ectopically expressed *licD1* in the mutant lacking both *licD1* and *licD2* (SPH135), cells grown in the presence (2 μM) or absence of ComS* were compared by differential interference contrast (DIC) microscopy. Cells were fixed in a paraformaldehyde-glutaraldehyde solution (2.5:1; 7.5%:0.018% [wt/vol] in PBS [pH 7.2]). After fixation on ice for 30 to 60 min, the cells were examined using a Zeiss LSM 700 microscope.

Transcriptional analyses. Pneumococcal RNA was isolated by using the RNeasy kit from Qiagen. Cells were grown to an OD_{550} of 0.1 and then cultures were split in two; one cell culture was induced with ComS* (2 μM) for 30 and 60 min, while the parallel culture was allowed to grow without ComS*. Cells from 10-ml culture aliquots of induced and noninduced cells were collected by centrifugation at 4°C at 4,000 × *g* for 5 min. The cell pellets were first treated with RNAlater (Qiagen) to stabilize the RNA and then suspended in a mixture of 700 μl RLT buffer (Qiagen) and 500 μl chloroform. This mixture was transferred to a FastPrep tube (MP Biomedicals Europe) containing 0.5 g of ≤106-μm acid-washed glass beads (Sigma). The cells were lysed in a FastPrep-24 apparatus (MP Biomedicals Europe) at 6.5 m s^{-1} five times for 30 s each. Insoluble cell debris was removed by centrifugation at 16,000 × *g* for 5 min before the water phase was transferred to a fresh Eppendorf tube and mixed with 500 μl ethanol. RNA from this mixture was isolated using an RNeasy minikit column as described by the manufacturer (Qiagen).

Prior to cDNA synthesis, the RNA was treated with DNase I to remove unwanted genomic DNA as follows: 20 μg RNA (the RNA concentration in samples varied between 1 and 2 μg/μl) was mixed with 1 μl RNaseOut (Invitrogen) and 10 μl DNase I (RNase-free; Qiagen) in 70 μl RDD buffer (Qiagen) and incubated at 37°C for 30 min. After DNA digestion, the DNase I was removed by performing a phenol-chloroform extraction. Phenol (pH 7.6), chloroform, and sample were mixed in a 1:1:1 ratio and vortexed for 1 min. Following centrifugation at 10,000 × *g* for 5 min, and RNA in the water phase was precipitated in 960 μl ethanol containing 40 μl 3 M sodium acetate and left overnight at −20°C. The precipitated RNA was collected by centrifugation at 16,000 × *g* for 30 min at 4°C, washed with 70% ice-cold ethanol, air dried, and resolved in RNase-free water. cDNA was synthesized from 1 μg DNase I-treated RNA using the Superscript III reverse transcriptase kit as described by the manufacturer (Invitrogen). Real-time PCR was carried out using the StepOnePlus real-time PCR system and the SYBR Green PCR master mix from Applied Biosystems. Twenty-five nanograms of cDNA was used as template, together with the primer pairs 16sF and 16sR targeting the 16S rRNA gene, lucF and lucR targeting the *luc* gene, and cbpDF and cbpDR targeting *cbpD*. The level of 16S rRNA expression was used to normalize the data for the different samples, and the relative levels of *luc* were determined based on the change in *luc* expression relative to the level of *cbpD*.

RESULTS

Establishment of the ComRS system in the pneumococcal genome. In a recent study, Fontaine et al. (9) proposed a simple model for induction of natural transformation in *S. thermophilus*. According to their model the Ami system transports the ComS* peptide into the cytoplasm, where it directly interacts with the ComR transcriptional activator. Upon binding of ComS*, ComR is thought to undergo a conformational change that allows it to bind to the ECom box in the *comX* promoter and activate transcription of the *comX* gene. Previous studies have shown that the alternative sigma factor ComX controls the competence regulon in *S. thermophilus*, i.e., the genes involved in binding, uptake, and integration of exogenous DNA (2). How the ComS* precursor is processed and how the mature peptide pheromone is exported from the cell are not known.

To determine if the proposed model is correct, we inserted the *comR* gene and the *comX* promoter fused to the firefly luciferase gene (P_{comX}::*luc*) into neutral sites of the pneumococcal genome. The *comR* gene, including its own promoter (Fig. 1), was inserted between the *amiF* and *treR* genes, whereas the P_{comX}::*luc* fragment was inserted between *cpsO*

and *cpsN*. Due to a nonfunctional capsule locus, the *S. pneumoniae* strain R6 is unencapsulated. Insertion of a foreign fragment between *cpsO* and *cpsN* should therefore have no biological consequences. We did not transfer the ComS* uptake system to *S. pneumoniae*, as we considered it likely that the native oligopeptide permease is able to translocate ComS* across the pneumococcal membrane. To evaluate the performance of the ComRS expression system, the resulting pneumococcal mutant strain (SPH126), carrying the *comR* gene and the P_{comX}::*luc* fusion, was grown in 96-well Corning NBS plates at 37°C inside a Fluostar Optima luminometer. When an OD_{492} of 0.1 was reached, ComS* was added at concentrations ranging from 0 to 16 μM. In cultures treated with ComS*, light emission started to rise above background levels 10 min postinduction and continued to increase for about 1 h. Based on our previous experience with the *luc* reporter and the Fluostar Optima luminometer, the observed maximum level of luminescence was relatively weak. Hence, before evaluating the ComRS system for its potential use in gene depletion studies, we decided to investigate whether it was possible to improve the level of ComS*-induced expression of target genes.

Improved expression of the *luc* reporter. Uptake of ComS* in *S. thermophilus* LMD-9 relies on a multisubunit ABC-type transporter consisting of two integral membrane proteins and two cognate ATP-binding proteins located on the cytoplasmic side of the membrane. In addition, the genome of strain LMD-9 encodes two extracellular oligopeptide-binding proteins that are responsible for capturing peptides from the external medium. Gardan et al. (10) showed that one of the oligopeptide-binding proteins, AmiA3, translocates ComS* more efficiently than the other. A possible explanation for the modest ComS*-induced *luc* expression observed with the SPH126 strain, therefore, was that the native oligopeptide-binding protein of *S. pneumoniae* has low affinity for ComS*, causing inefficient uptake of ComS* and reduced *luc* expression. We tested this possibility by incorporating a copy of the *amiA3* gene and its promoter into the genome of the SPH126 strain between the *cpsO* and spr0325 genes. The resulting mutant strain (SPH128) produced the same amount of light as the parental strain, demonstrating that addition of the *amiA3* gene did not contribute to increased *luc* expression (data not shown).

Another factor that might limit ComS*-induced *luc* expression is the amount of ComR produced in the pneumococcal cell. It is quite conceivable that the native *comR* promoter functions poorly in *S. pneumoniae* and that ComR therefore is weakly expressed. The synthetic constitutive promoter P_1 (14) was therefore introduced upstream of the native *comR* promoter (Fig. 1), giving rise to the SPH130 strain. Characterization of this strain revealed that it emitted about 5-fold more light than the parental strain when equal amounts of ComS* were used to induce *luc* expression (data not shown).

The level of *luc* expression can be modulated by ComS*. The properties of the ComRS system were further investigated by subjecting the SPH130 strain to different levels of ComS*. The results presented in Fig. 2A show that no light emission above background level was detected by the Fluostar Optima luminometer in uninduced cultures. Interestingly, addition of different concentrations of ComS* gave rise to different levels of luciferase activity, demonstrating that expression of target genes driven by P_{comX} can be controlled by varying the peptide pheromone concentration in the growth medium (Fig. 2A). This dose-dependent activation of *luc* expression was also clearly seen in the Western analysis depicted in Fig. 2B. Maximum luciferase production was obtained with external ComS* concentrations in the range of 1 to 2 μM. In the presence of ComS*, expression of the *luc* gene persisted throughout the growth phase but dropped as the culture approached stationary phase (Fig. 2A). Importantly, our results showed that transfer of SPH130 cells from ComS*-containing medium (2 μM) to ComS*-free medium brought about a gradual reduction in light emission after a lag period of 45 to 60 min (Fig. 3). This result was very promising with respect to the potential use of the ComRS system as a tool for gene depletion studies.

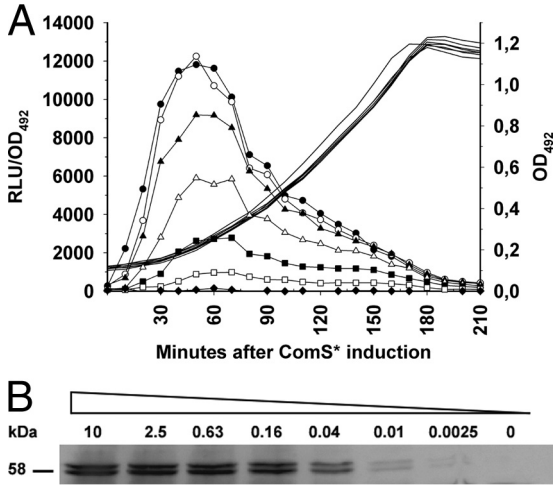

FIG. 2. Bioluminescence (A) and Western analyses (B) showing the level of Luc expression after subjecting SPH130 cells to various concentrations of ComS*. (A) SPH130 cell cultures were induced at an OD_{492} of 0.1 with the following concentrations of ComS*: 0 (◆), 0.08 μM (□), 0.16 μM (■), 0.31 μM (Δ), 0.63 μM (▲), 1.25 μM (○), or 10 μM (●). Luminescence relative to cell density (in relative light units [RLU]/OD_{492}) is indicated by lines with symbols, while lines without symbols indicate bacterial growth. Concentrations of ComS* higher than 1 to 2 μM did not increase the emitted light intensity, suggesting a saturation of the system, whereas induction with lower concentrations of ComS* displayed less production of light in a dose-dependent manner. The data shown are from a representative experiment of several replicates. (B) Detection of the luciferase enzyme by Western analysis after induction for 1 h with different concentrations of ComS*. ComS* was added to SPH130 cell cultures at an OD_{492} of 0.1. Luciferase was detected by using a polyclonal antiluciferase antibody produced in rabbits. The concentrations of ComS* used are indicated in μM above the respective protein bands. The bands appearing immediately below the full-size luciferase bands represent degradation products. Our results showed that the luciferase enzyme is very unstable in *S. pneumoniae*.

Depletion of LicD1. As mentioned in the introduction, previous investigations had suggested that the pneumococcus can grow normally with a functional *licD1* or *licD2* gene but will not survive deletion of both. It was shown by Zhang and coworkers (26) that the *licD2* gene can be readily knocked out by insertion-duplication mutation, while this strategy failed with the *licD1* gene. The reason for this is probably that insertion of a plasmid in the *licD1* gene has a polar effect on *licD2* expression. To determine whether the ComRS system is suitable for

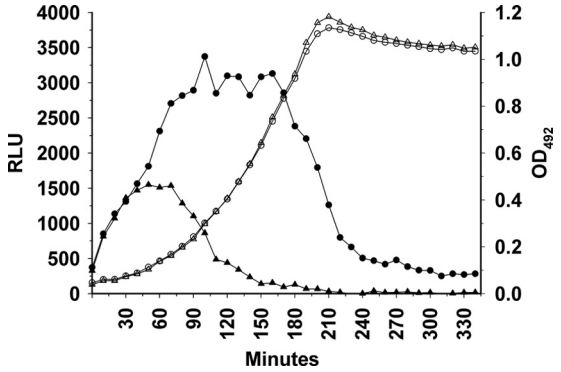

FIG. 3. Decay of luciferase activity over time after removal of ComS* from the growth medium. The SPH130 strain was grown either in the presence of 2 μM ComS* (circles) or in the absence of ComS* (triangles). Bacterial growth is indicated by open symbols, whereas luminescence is indicated by filled symbols. After shifting the cells at time zero from a medium containing 2 μM ComS* to a ComS*-free medium, it took about 70 min before the luciferase activity started to decline (▲). In the parallel culture grown in the presence of ComS*, the luciferase activity started to decline as the culture approached stationary phase (●).

depletion studies of lethal genes, we decided to construct a *licD1 licD2* double-knockout mutant. First, a PCR fragment corresponding to the *licD1* gene was inserted behind the P_{comX} promoter essentially as described for the *luc* gene above. Then, the resulting strain (SPH132) was transformed with a PCR fragment designed to replace *licD1* and most of *licD2* with the Janus cassette (24), giving rise to SPH135. During transformation and subsequent selection of transformants on agar plates containing kanamycin, 2 μM ComS* was added to the growth medium. After incubation at 37°C, overnight colonies were picked and seeded into medium containing 2 μM ComS*. Analysis of the mutants by PCR and sequencing showed that the Janus cassette had been inserted correctly. As a control experiment, wild-type cells were transformed in parallel with the same fragment. In this case, no transformants were obtained. These results show that *S. pneumoniae* can survive with only *licD1* but is not able to grow in the absence of both *licD* genes. They also showed that the P_{comX} promoter is able to drive expression of an essential gene after the native gene has been deleted.

Next we wanted to determine if removal of ComS* from the growth medium would deplete the amount of LicD1 in the pneumococcal cells to a physiologically critical level. A culture of the SPH135 mutant grown in C medium containing 2 μM ComS* was pelleted and washed once in plain C medium. After washing, the bacterial pellet was suspended in ComS*-free C medium to an OD_{492} of 0.05 and then serially diluted 2-fold in the same medium in a 96-well Corning NBS plate with a clear bottom. Following dilution of the cells, 2 μM ComS* was added to the wells in one row (Fig. 4A), while the wells in the parallel row contained no ComS* (Fig. 4B). The plate was incubated at 37°C in an Optima Fluostar luminometer for 11 h, during which the OD_{492} was measured automatically by the luminometer every 10 min. The results showed that cells cultivated in the absence of ComS* grew normally for about 5 h, after which they started to lag behind the positive control. After about 7 to 8 h, the cultures stopped growing and started

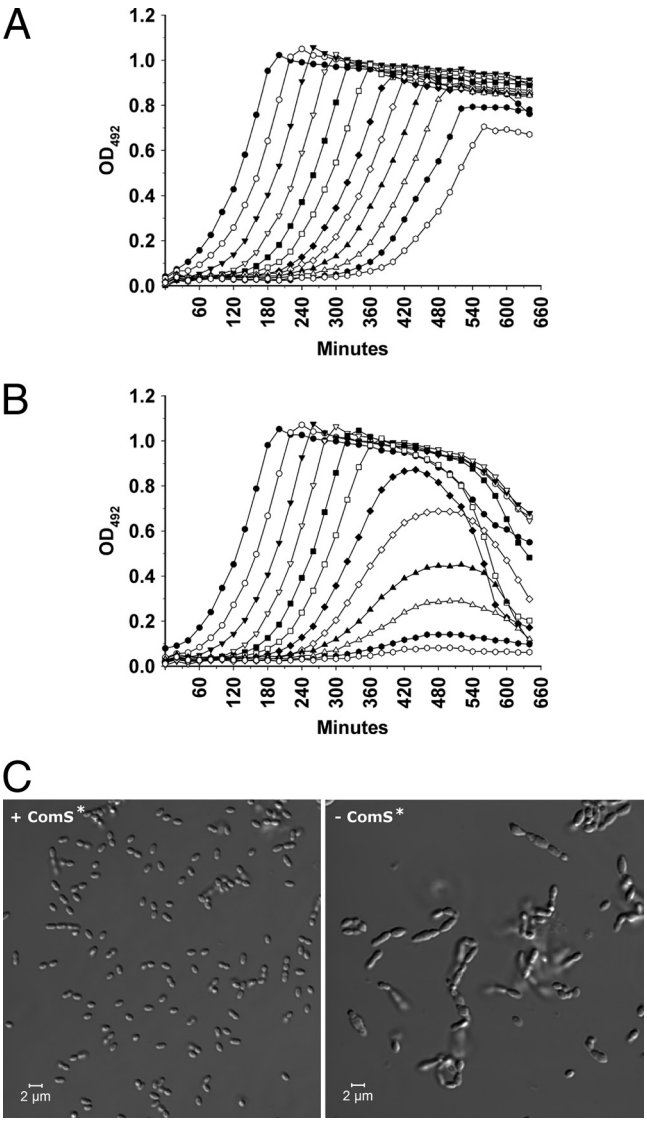

FIG. 4. Effects of *licD1* depletion on growth and morphology of SPH135 cultures. (A) A culture of SPH135 cells grown to an OD_{492} of 0.3 in C medium supplemented with 2 μM ComS* was pelleted, washed once in plain C medium, and resuspended to an OD_{492} of 0.05 in fresh C medium containing 2 μM ComS*. Then, the culture was 2-fold diluted in the same medium in a 96-well plate and incubated in a Fluostar Optima luminometer at 37°C for 11 h. (B) The same culture of SPH135 cells was washed and resuspended in ComS*-free medium but otherwise treated as describe for panel A. In cells growing in the presence of ComS* (A), ectopic expression of *licD1* is driven by the ComRS system. In cells growing in ComS*-free medium (B), ectopic expression of *licD1* is gradually reduced. About 5 h after the cells were shifted from a ComS*-containing to a ComS*-free medium, growth of LicD1-depleted cells started to slow down. A few hours later the growth stopped completely, and the cells started to lyse (B). The data shown are from a representative experiment of several replicates. (C) Examination of *licD1*-proficient (+ComS*) and *licD1*-deficient (−ComS*) SPH135 cells by DIC microscopy. Samples of *licD1*-proficient and *licD1*-deficient cells were collected at the transition between logarithmic and stationary phases (at 480 min) from the cultures represented by open triangles (see panels A and B). The pictures shown are representative several independent experiments. The morphology of SPH135 cells grown in the presence of ComS* was indistinguishable from that of wild-type pneumococci, while the morphology of *licD1*-depleted cells was clearly abnormal.

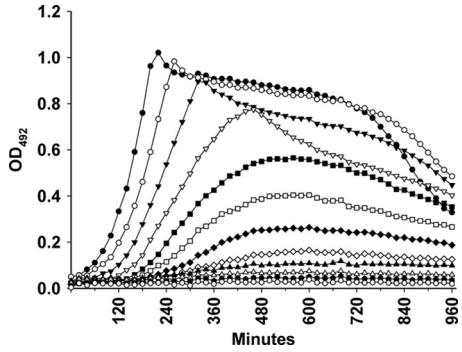

FIG. 5. Depletion of the Kan^r gene makes the SPH136 mutant sensitive to kanamycin. SPH136 cell cultures were grown in C medium containing 2 μM ComS* until they reached an OD_{492} of 0.3. Then, they were pelleted, washed once, and resuspended to an OD_{492} of 0.05 in ComS*-free C medium containing kanamycin (400 μg ml^{-1}). The resuspended cells were 2-fold diluted in the same medium in a 96-well plate and incubated in a Fluostar Optima luminometer at 37°C for 16 h. Growth (measured as the OD_{492}) was determined automatically by the luminometer at 10-min intervals. As Kan^r was depleted over time, the growth rate of the SPH136 cells gradually slowed down. After being cultivated for about 8 h in ComS*-free medium, their growth was completely inhibited by kanamycin.

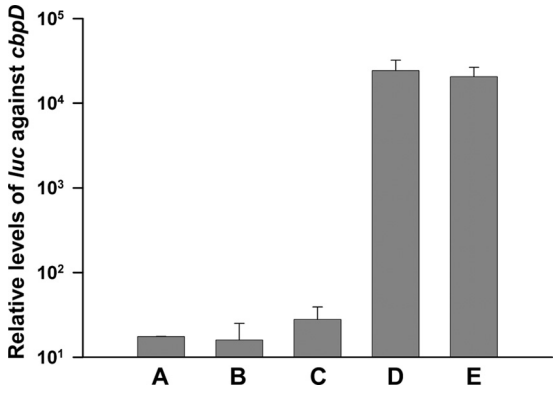

FIG. 6. Expression levels of *luc* relative to the expression levels of *cbpD* measured by real-time RT-PCR. The late competence gene *cbpD* is only expressed in cells that are competent for natural genetic transformation. (A) Background transcription of *luc* was examined in SPH130 cells grown without ComS* to an OD_{550} of 0.1. (B and C) Additional samples from the same culture were collected 30 (B) and 60 min (C) later and analyzed in the same way. The results showed that the background expression of *luc* in uninduced cells was 10 to 30 times higher than the background expression of *cbpD* in noncompetent cells. Comparison of *luc* expression levels in cells induced with 2 μM ComS* for 30 and 60 min (D and E) with those of noninduced cells run in parallel (B and C) showed that addition of ComS* increased *luc* expression about 1,500-fold.

to lyse. Light microscopic examination of LicD1-depleted pneumococcal cultures exhibiting decreasing growth rates revealed morphologically abnormal bacteria (Fig. 4C). The cells were much larger than normal, and many had a grossly deformed elongated shape. In addition, the LicD1-depleted cells grew in short chains.

To further test the performance of the ComRS system, we introduced a kanamycin resistance gene (*aacA-aphD*) from the plasmid pFW13 (22) behind the P_{comX} promoter (SPH136) to show that depletion of this Kan^r gene by removal of ComS* rendered the cells sensitive to kanamycin (400 μg ml^{-1}). In this context, the Kan^r gene can be defined as essential for SPH136. As Fig. 5 shows, the cells became highly sensitive to kanamycin during the Kan^r depletion experiment. ComS*-induced SPH136, however, remained resistant to kanamycin and grew normally to stationary phase (data not shown).

How tightly regulated is the *comX* promoter? The time it took before removal of ComS* from the medium had an effect on the growth rate of the SPH135 mutant was longer than expected. We suspected that the long reaction time was partially caused by low-level background transcription of the *licD1* gene in the absence of the ComS* inducer. Such background transcription might originate from the P_{comX} promoter itself, or from a promoter located further upstream of the *licD1* gene. To investigate these matters, we performed transcriptional analyses on the SPH130 strain, which contains the *luc* gene inserted behind the P_{comX} promoter. The level of *luc* transcription was examined before and after induction with the ComS* peptide (2 μM) and compared with the expression level of the late competence gene *cbpD*. Transcription of *cbpD*, which is controlled by the alternative sigma factor ComX, is shut off in noncompetent cells (15). By real-time reverse transcription-PCR (RT-PCR), it was demonstrated that the basal level of *luc* transcripts was 10 to 30 times higher than the corresponding level of *cbpD* transcripts (Fig. 6). In addition, it was shown that *luc* expression in cultures treated with 2 μM ComS* increased approximately 1,500-fold compared to uninduced cultures. Further experiments are needed to determine whether the observed basal level of *luc* expression originates from the P_{comX} promoter itself or from regions upstream of this promoter.

DISCUSSION

In the present study we exploited the transcriptional activator ComR, its inducer ComS*, and the P_{comX} promoter from *S. thermophilus* to construct a gene depletion system for use in *S. pneumoniae*. The system also represents a useful tool for controlled expression of selected genes at physiological levels. However, since the P_{comX} promoter appears to be only moderately strong when fully induced by ComS*, the ComRS system is probably not suitable for high-level overexpression of genes. Two other gene depletion systems devised for use in *S. pneumoniae* have been described. One of them, which was developed by Chan et al. (3), is based on the fucose-regulated promoter P_{fcsK}. In this system, a PCR-generated cassette consisting of the P_{fcsK} promoter, a selectable antibiotic resistance marker, and appropriate flanking regions is introduced upstream of the target gene by homologous double-crossover recombination. A disadvantage of this strategy is that codepletion of downstream genes will take place if they are located on the same transcription unit as the target gene. Since all *S. pneumoniae* strains tested so far have been unable to grow on fucose as a sole carbon and energy source, the role of this sugar in pneumococcal metabolism remains unclear (3). An alternative method based on the Zn^{2+}-inducible promoter P_{czcD} was described recently (7, 17). To perform a gene depletion experiment with the Zn^{2+} system, a DNA cassette containing a selectable antibiotic resistance marker and the gene of interest under the control of the P_{czcD} promoter was inserted into the

pneumococcal *bgaA* locus via homologous double-crossover recombination. Then, in the presence of the relatively high Zn^{2+} concentration needed to drive ectopic expression of the target gene, the native copy of this gene can be deleted from the genome by replacing it with another antibiotic resistance marker. Depletion of the selected gene takes place when the Zn^{2+} concentration in the medium is reduced. As zinc plays an important role in pneumococcal physiology, the expression and function of a number of gene products are potentially affected by the levels of this metal ion (6, 16, 17, 21). The Zn^{2+} depletion system should therefore be used with caution. In contrast to the gene depletion techniques described above, which are based on the native P_{fcsK} and P_{czcD} promoters, the ComRS system is of heterologous origin. As no close homologues of the ComRS proteins are encoded in the pneumococcal genome, it is unlikely that the presence of the ComRS system interferes with the normal physiology of the cell.

When the SPH130 mutant was shifted from a medium containing 2 μM ComS* to a ComS*-free medium, it took about 1 h before Luc-generated light emission leveled off and started to decline (Fig. 3). However, following the same procedure, it took about 5 h before depletion of LicD1 affected the growth rate of the SPH135 mutant (Fig. 4B). To determine whether it was possible to reduce the response time, we tried to grow the SPH135 mutant at a lower concentration of ComS* before shifting the culture to ComS*-free medium. It turned out that 0.02 μM ComS* was sufficient to result in a normal growth rate and morphology. In this case the response time was reduced to 3 to 4 h (result not shown). This result indicates that residual ComS* is removed relatively slowly in cultures that have been shifted to ComS*-free medium. A possible explanation is that intracellular ComS* is highly stable and is removed from the cytoplasm very slowly, either by peptidases or by dilution as the cells grow and divide. Whatever the correct explanation, our results demonstrate that gene depletion can easily be achieved using the ComRS system. In fact, the delayed response might be an advantage when performing depletion studies of essential genes. A rapid decrease in gene expression would probably result in growth arrest and cell death before any distinguishable phenotype had time to develop. A slow reduction, on the other hand, leads to a gradual buildup of stress that allows the cells to develop phenotypic changes before they stop growing. It should be noted, though, that the observed background transcription could cause problems if the gene selected for depletion studies were expressed at a very low level. We hope to eliminate this potential problem in future studies by identifying and removing the sequence element(s) responsible for the observed background transcription.

Depletion of LicD1 in the SPH135 mutant, which lacks the native *licD1* and *licD2* genes, caused striking morphological alterations. Almost all of the LicD1-depleted SPH135 cells grew to a much larger size than corresponding cells grown in the presence of ComS*. This result indicates that LicD1-deficient cells have lost the ability to divide normally. This view is substantiated by other morphological abnormalities characteristic of SPH135 cells grown in the absence of ComS*. The LicD1-deficient cells are morphologically heterogeneous and include some very long, misshapen cells. The elongated cells are evidently able to incorporate new peptidoglycan into their cell walls, but they struggle to divide. Since the TacF flippase is strictly specific for choline-containing subunits (5), depletion of LicD1 will reduce the amount of WTA and LTA in the cell wall of SPH135 cells. Thus, the observed morphological abnormalities are most likely caused by suboptimal levels of WTA and/or LTA. However, it is possible that peptidoglycan synthesis could also be affected by severe LicD1 depletion. The reason for this is that teichoic acid as well as peptidoglycan synthesis depend on the membrane-anchored undecaprenyl carrier lipid. During LicD1 depletion, undecaprenyl-linked choline-free teichoic acid precursors are trapped at the inside of the cytoplasmic membrane. This might exhaust the supply of free carrier that is available for peptidoglycan synthesis. However, the fact that LicD1 depletion results in oversized cells indicates that peptidoglycan synthesis is not critically affected. The observed morphological abnormalities are therefore most likely caused by reduced incorporation of teichoic acid in the pneumococcal cell wall.

ACKNOWLEDGMENT

This work was supported by The Research Council of Norway.

REFERENCES

1. **Baur, S., J. Marles-Wright, S. Buckenmaier, R. J. Lewis, and W. Vollmer.** 2009. Synthesis of CDP-activated ribitol for teichoic acid precursors in *Streptococcus pneumoniae*. J. Bacteriol. **191:**1200–1210.
2. **Blomqvist, T., H. Steinmoen, and L. S. Håvarstein.** 2006. Natural genetic transformation: a novel tool for efficient genetic engineering of the dairy bacterium *Streptococcus thermophilus*. Appl. Environ. Microbiol. **72:**6751–6756.
3. **Chan, P. F., et al.** 2003. Characterization of a novel fucose-regulated promoter (P_{fcsK}) suitable for gene essentiality and antibacterial mode-of-action studies in *Streptococcus pneumoniae*. J. Bacteriol. **185:**2051–2058.
4. **Chastanet, A., M. Prudhomme, J. P. Claverys, and T. Msadek.** 2001. Regulation of *Streptococcus pneumoniae clp* genes and their role in competence development and stress survival. J. Bacteriol. **183:**7295–7307.
5. **Damjanovic, M., A. S. Kharat, A. Eberhardt, A. Tomasz, and W. Vollmer.** 2007. The essential *tacF* gene is responsible for the choline-dependent growth phenotype of *Streptococcus pneumoniae*. J. Bacteriol. **189:**7105–7111.
6. **Dintilhac, A., G. Alloing, C. Granadel, and J. P. Claverys.** 1997. Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. Mol. Microbiol. **25:**727–739.
7. **Eberhardt, A., L. J. Wu, J. Errington, W. Vollmer, and J. W. Veening.** 2009. Cellular localization of choline-utilization proteins in *Streptococcus pneumoniae* using a novel fluorescent reporter system. Mol. Microbiol. **74:**395–408.
8. **Fischer, W., T. Behr, R. Hartmann, J. Peter-Katalinic, and H. Egge.** 1993. Teichoic acid and lipoteichoic acid of *Streptococcus pneumoniae* possess identical chain structures. A reinvestigation of teichoic acid (C polysaccharide). Eur. J. Biochem. **215:**851–857.
9. **Fontaine, L., et al.** 2010. A novel pheromone quorum-sensing system controls the development of natural competence in *Streptococcus thermophilus* and *Streptococcus salivarius*. J. Bacteriol. **192:**1444–1454.
10. **Gardan, R., C. Besset, A. Guillot, C. Gitton, and V. Monnet.** 2009. The oligopeptide transport system is essential for the development of natural competence in *Streptococcus thermophilus* strain LMD-9. J. Bacteriol. **191:**4647–4655.
11. **Håvarstein, L. S., G. Coomaraswami, and D. A. Morrison.** 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. U. S. A. **92:**11140–11144.
12. **Higuchi, R., C. H. von Beroldingen, G. F. Sensabaugh, and H. A. Erlich.** 1988. DNA-typing from single hairs. Nature **332:**543–546.
13. **Johnsborg, O., V. Eldholm, M. L. Bjørnstad, and L. S. Håvarstein.** 2008. A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae*. Mol. Microbiol. **69:**245–253.
14. **Johnsborg, O., and L. S. Håvarstein.** 2009. Pneumococcal LytR, a protein from the LytR-CpsA-Psr family, is essential for normal septum formation in *Streptococcus pneumoniae*. J. Bacteriol. **191:**5859–5864.
15. **Kausmally, L., O. Johnsborg, M. Lunde, E. Knutsen, and L. S. Håvarstein.** 2005. Choline-binding protein D (CbpD) in *Streptococcus pneumoniae* is essential for competence-induced cell lysis. J. Bacteriol. **187:**4338–4345.
16. **Kloosterman, T. G., M. M. van der Kooi-Pol, J. J. E. Bijlsma, and O. P.**

Kuipers. 2007. The novel transcriptional regulator SczA mediates protection against Zn^{2+} stress by activation of the Zn^{2+}-resistance gene *czcD* in *Streptococcus pneumoniae*. Mol. Microbiol. **65:**1049–1063.

17. **Kloosterman, T. G., R. M. Witwicki, M. M. van der Kooi-Pol, J. J. E. Bijlsma, and O. P. Kuipers.** 2008. Opposite effects of Mn^{2+} and Zn^{2+} on PsaR-mediated expression of the virulence genes *pcpA*, *prtA*, and *psaBCA* of *Streptococcus pneumoniae*. J. Bacteriol. **190:**5382–5393.
18. **Lacks, S., and R. D. Hotchkiss.** 1960. A study of the genetic material determining an enzyme in pneumococcus. Biochim. Biophys. Acta **39:**508–518.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
20. **LeMessurier, K. S., A. D. Ogunniyi, and J. C. Paton.** 2006. Differential expression of key pneumococcal virulence genes *in vivo*. Microbiology **152:** 305–311.
21. **Ogunniyi, A. D., et al.** 2009. Pneumococcal histidine triad proteins are regulated by the Zn^{2+}-dependent repressor AdcR and inhibit complement deposition through the recruitment of complement factor H. FASEB J. **23:**731–738.
22. **Podbielski, A., B. Spellerberg, M. Woischnik, B. Pohl, and R. Lütticken.** 1996. Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). Gene **177:**137–147.
23. **Song, J. H., et al.** 2005. Identification of essential genes in *Streptococcus pneumoniae* by allelic replacement mutagenesis. Mol. Cell **19:**365–374.
24. **Sung, C. K., H. Li, J. P. Claverys, and D. A. Morrison.** 2001. An *rpsL* cassette, Janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. Appl. Environ. Microbiol. **67:**5190–5196.
25. **Thanassi, J. A., S. L. Hartman-Neumann, T. J. Dougherty, B. A. Dougherty, and M. J. Pucci.** 2002. Identification of 113 conserved essential genes using a high-throughput gene disruption system in *Streptococcus pneumoniae*. Nucleic Acids Res. **30:**3152–3162.
26. **Zhang, J. R., I. Idanpaan-Heikkila, W. Fischer, and E. I. Tuomanen.** 1999. Pneumococcal *licD2* gene is involved in phosphorylcholine metabolism. Mol. Microbiol. **31:**1477–1488.