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Two Distinct Functions of ComW in Stabilization and Activation of the Alternative Sigma Factor ComX in *Streptococcus pneumoniae*

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Natural genetic transformation in *Streptococcus pneumoniae* is controlled by a quorum-sensing system, which acts through the competence-stimulating peptide (CSP) for transient activation of genes required for competence. More than 100 genes have been identified as CSP regulated by use of DNA microarray analysis. One of the CSP-induced genes required for genetic competence is *comW*. As the expression of this gene depended on the regulator ComE, but not on the competence sigma factor ComX (σ^X), and as expression of several genes required for DNA processing was affected in a *comW* mutant, *comW* appears to be a new regulatory gene. Immunoblotting analysis showed that the amount of the σ^X protein is dependent on ComW, suggesting that ComW may be directly or indirectly involved in the accumulation of σ^X. As σ^X is stabilized in *clpP* mutants, a *comW* mutation was introduced into the *clpP* background to ask whether the synthesis of σ^X depends on ComW. The *clpP comW* double mutant accumulated an amount of σ^X higher (threefold) than that seen in the wild type but was not transformable, suggesting that while *comW* is not needed for σ^X synthesis, it acts both in stabilization of σ^X and in its activation. Modification of ComW with a histidine tag at its C or N terminus revealed that both amino and carboxyl termini are important for increasing the stability of σ^X, but only the N terminus is important for stimulating its activity.

Streptococcus pneumoniae (pneumococcus) is a naturally transformable bacterium that can become competent to take up exogenous DNA. At a certain cell density during the exponential growth phase, the sudden appearance of competence is controlled in part by a quorum-sensing system, which acts through a secreted pheromone to cause transient activation of many genes required for competence (10, 25). The pheromone is processed from a precursor (a product of *comC*) by a membrane transporter, ComAB, to release the mature 17-residue competence-stimulating peptide (CSP) (10, 11, 12, 25). When CSP accumulates in the medium, its presence is sensed by a receptor histidine kinase, ComD, and this sensor kinase probably phosphorylates the response regulator, ComE, activating the expression of *comAB*, *comCDE*, and several other operons (10, 16, 25, 26).

Operons under control of the response regulator ComE have an imperfect direct-repeat sequence in their promoter regions, and ComE appears to bind this target site to activate gene expression (37). Two identical copies of the *comX* gene (*comX1* and *comX2*) which depend on ComE for their CSP-induced expression (16, 26, 27) encode a competence-specific alternative sigma factor, σ^X. Expression of *comX* allows transcription of many genes encoding components of the machinery for DNA uptake and recombination and thus links the quorum-sensing signal to the DNA uptake pathway (16, 18, 20). Genes under its control share an 8-bp sequence in their promoter regions that is specifically required by σ^X-containing RNA polymerase (4, 8, 16, 20). Thus, genes involved in genetic transformation can be grouped into two classes by their modes of regulation: early genes are ComE dependent, and their mRNA reaches a maximum 7 to 8 min after CSP treatment, while late competence genes are also σ^X dependent, and their mRNA peaks at 10 to 11 min (1, 8, 26).

Several surveys, including a promoter-trap study (2), a gene array hybridization survey (28), a bioinformatics promoter sequence search (26), and DNA microarray surveys (26, 27), have identified ~180 CSP-responsive genes in *S. pneumoniae*. Although many of these genes are not directly required for transformation or competence induction (27), one of the CSP-induced genes that is required for competence is *comW*. As the induction of *comW* does not depend on σ^X, and as *comW* is essential for competence, Peterson et al. proposed that it might be a new regulatory gene (27). Recently, Luo et al. (19) reported that the ectopic coexpression of *comW* and *comX* leads to the induction of competence and the accumulation of σ^X in the absence of CSP treatment at 30°C, suggesting that ComW acts as a positive regulator of *comX*, at least under these experimental conditions.

Although *comW* was thus identified as a probable actor in the linkage between early and late competence genes, it is not known how it promotes the development of competence or what is the biological significance of this role. Here, we present evidence that ComW contributes to the stabilization of the alternative sigma factor σ^X against proteolysis. We also provide evidence that *comW* is required for full activity of σ^X in directing transcription of late competence genes.

MATERIALS AND METHODS

Bacterial strains, media, antibiotics, and DNA sources. The pneumococcal strains and plasmids used in this study are listed in Tables 1 and 2. CP1250 (25) was used as a transformation recipient to create all new pneumococcal mutations

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TABLE 1. Pneumococcal strains used in this study

Strain	Description	Source[a]
CP1250	*S. pneumoniae* Rx derivative; low β-galactosidase activity; *hex malM511 str-1 bgl-1* Hex$^-$ Mal- Sm^r Bga$^-$	25
CP1500	*hex nov-r1 bry-r str-1 ery-r1 ery-r2* Nov^r	5
CP1359	CP1250, but *clpP*::*tet* Tet^r	17
CP1376	CP1250, but *comW*::KANT Kan^r	27
CP1548	CP1250, but *cglA*::pEVP3 Cm^r	16
CP1601	CP1250, but *celB*::pEVP3 Cm^r	16
CP1714	*comW*::KANT *ssbB*::pEVP3::*ssbB*$^+$ Cm^r Kan^r	CP1376 × CPM7
CP1718	*cglA*::pEVP3 Cm^r Kan^r	CP1376 × CP1548
CP1719	*celB*::pEVP3 Cm^r Kan^r	CP1376 × CP1601
CP1721	CP1250, but *comW*::pEVP3::*comW*$^+$ Cm^r	pCKS03 × CP1250
CP1723	*comW*::KANT *comX1*::*erm comX2*::*Tet* Erm^r Kan^r Tet^r	CP1376 × CPM8
CP1724	*comW*::KANT *comX1*::pEVP3::*comX1*$^+$ Cm^r Kan^r	CP1376 × CPM3
CP1731	CP1721, but *comE*::*erm* Erm^r Cm^r	This study
CP1802	CP1250, but C terminus-V5-H6-ComW-pEVP3 Cm^r	pCKS07 × CP1250
CP1805	CP1250, but N terminus-H6-Xa-ComW-KANT Kan^r	This study
CP1815	*clpP*::*tet comW*::KANT Kan^r Tet^r	CP1376 × CP1359
CP1816	*clpP*::*tet* N-H6-Xa-ComW-KANT Kan^r Tet^r	CP1359 × CP1805
CP1820	*comW*::KANT *clpP*::*tet cglA*::pEVP3 Cm^r Kan^r Tet^r	CP1376 × CP1821
CP1821	*clpP*::*tet cglA*::pEVP3 Cm^r Tet^r	CP1548 × CP1359
CP1822	*comW*::KANT *clpP*::*tet celB*::pEVP3 Cm^r Kan^r Tet^r	CP1376 × CP1823
CP1823	*clpP*::*Tet celB*::pEVP3 Cm^r Tet^r	CP1601 × CP1359
CP1850	*clpP*::*Tet* C-V5-H6-ComW-pEVP3 Cm^r Tet^r	CP1359 × CP1802
CP1851	CP1250, but *clpE*::*erm* Erm^r	This study
CP1852	*comW*::KANT *clpE*::*erm clpP*::*tet* Erm^r Kan^r Tet^r	CP1376 × CP1853
CP1853	*clpE*::*erm clpP*::*tet* Erm^r Tet^r	CP1359 × CP1851
CP1854	*comW*::KANT *clpE*::*Erm* Erm^r Kan^r	CP1376 × CP1851
CPM3	CP1250, but *comX1*::pEVP3::*comX1*$^+$ Cm^r	16
CPM4	CP1250, but *comX1*::*Erm comX2*::pEVP3 Cm^r Erm^r	16
CPM7	CP1250, but *ssbB*::pEVP3::*ssbB*$^+$ Cm^r	16
CPM8	CP1250, but *comX1*::*Erm comX2*::*tet* Erm^r Tet^r	16

[a] Construction of a strain by transformation between two different mutant strains is shown as DNA × recipient.

and *lacZ* fusions. *Escherichia coli* strain BL21(DE3)/pLysS [F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)/pLysS] was the host for plasmids pCR T7/CT TOPO (Invitrogen, Carlsbad, CA) and pET16b (Novagen, Madison, WI). Complete CAT medium (15) and Luria-Bertani (LB) medium (3) were used for pneumococcal and *E. coli* cultures, respectively. DNA from strain CP1500 (5) was used as the donor for transformation assays. Primers used for constructing mutations in this study, listed in Table 2, were obtained from QIAGEN (Valencia, CA). Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol (Cm), 34 μg ml^{-1} for *E. coli* and 2.5 μg ml^{-1} for *S. pneumoniae*; erythromycin (Erm), 0.25 μg ml^{-1}; kanamycin (Kan), 200 μg ml^{-1}; novobiocin (Nov), 2.5 μg ml^{-1}; tetracycline (Tet), 0.25 μg ml^{-1}.

Construction of new pneumococcal strains. To create the *comW lacZ* fusion strain CP1721, a *comW* fragment was prepared by PCR using the primer pair CKS24 and CKS25. This amplicon and plasmid pEVP3 were digested with XbaI and BamHI (Invitrogen), ligated using T4 DNA ligase (MBI Fermentas, Hanover, MD), and introduced into *E. coli* DH5α. A recombinant plasmid (pCKS03) from a single Cm^r colony was then transferred to competent cells of CP1250. A Cm^r transformant was selected, and full circular integration of pCKS03 was verified by amplification and sequencing of new junction fragments. A similar strategy was used to create strain CP1802, expressing a C-terminally histidine-tagged ComW. Once pCKS01 was constructed in *E. coli* BL21(DE3)/pLysS using a *comW* PCR fragment (primers CKS33 and CKS34) and plasmid pCR T7/CT TOPO, the gene fusion encoding a His-tagged ComW was amplified from it using the primer pair CKS107 (XbaI) and CKS108 (BamHI). This amplicon was cloned into plasmid pEVP3, and the structure of the product (pCKS07) was verified by sequencing of new junctions. After transforming strain CP1250 with pCKS07 and verifying the expected insertion structure by PCR, a Cm^r colony was selected for further study and named CP1802.

To construct strain CP1805, expressing N-terminally histidine-tagged ComW, *comW* (amplified with primers CKS109 and CKS110) was fused to the N-terminal histidine tag-encoding sequence in plasmid pET16b (using NdeI and BamHI sites). From the resulting plasmid, pCKS02, the gene encoding N-His-tagged ComW was amplified using primers CKS116 (XbaI) and CKS110. PCR ligation mutagenesis was used to introduce the gene encoding the N-His-tagged protein with a ComE-responsive promoter into CP1250. The promoter sequence of *comW* (primers CKS114 [ApaI] and CKS115 [XbaI]) and a Kan resistance cassette (KANT; primers DAM303 [ApaI] and DAM304 [BamHI]) were amplified from chromosomal DNAs of CP1250 and plasmid pR410 (33), respectively. A fragment (UP fragment) upstream of *comW* was also amplified using primers DAM497 and CKS113 (BamHI). All four fragments (UP fragment, KANT, ComE promoter, and N-His-*comW*) were digested by the corresponding restriction enzymes, ligated, and introduced into CP1250. A Kan^r colony resulting from targeted recombination was retained as CP1805, and the structure of the chimeric locus was verified by PCR and sequencing of all three new junctions. Other new pneumococcal strains were made by transformation crosses, as indicated in Table 1.

Beta-galactosidase activity assay. For assay of *lacZ* activity in CP1724, CPM3, and CPM4, cells were grown in complete CAT medium supplemented with 8 mM HCl to avoid endogenous induction of competence. Optical density at 550 nm (OD_{550}) was determined in 18-mm-diameter round cuvettes in the Coleman Jr II colorimeter. At an OD_{550} of 0.05, each culture was induced by addition of CSP-1 (200 ng ml^{-1}), bovine serum albumin (BSA) (0.2%), and $CaCl_2$ (0.5 mM), and 1-ml samples were withdrawn at 5-min or 10-min intervals for 60 min. Each sample was lysed with 0.2% Triton X-100 at 37°C for 10 min, mixed with 1 ml of substrate buffer (1.6 mg *o*-nitrophenyl-β-D-galactopyranoside [ONPG], 20 mM sodium phosphate [16.4 mM Na_2HPO_4 and 3.6 mM NaH_2PO_4; pH 7.5], 20 mM NaOH, 2 mM $MgCl_2$, and 90 mM β-mercaptoethanol) (30). After 40 min at 37°C and addition of 1 ml of 1 M Na_2CO_3, the optical density was measured at 420 nm. For other *lacZ* fusion strains, cells were grown to an OD_{550} of 0.05, and both a noncompetent culture and a parallel sample of the same culture treated with CSP for 20 min were lysed and assayed for beta-galactosidase as described above.

Immunoblotting. Cells were grown in complete CAT medium containing 8 mM HCl to an OD_{550} of 0.05. A 10-ml sample was chilled and centrifuged at 10,000 rpm for 10 min as an uninduced control. For the induced samples, each culture was treated with 200 ng ml^{-1} of CSP, and 10-ml samples were withdrawn at the indicated times. The samples were chilled rapidly in 1-liter steel beakers on

TABLE 2. Plasmids and oligonucleotide primers used in this study

Plasmid/primer	Description/sequence (5′-3′)	Source or location/use
Plasmids		
pCKS01	C-V5-H6-*comW* in plasmid pCRT7/CT TOPO	This study
pCKS02	N-H6-Xa-*comW* in plasmid pET16b	This study
pCKS03	*comW* in plasmid pEVP3	This study
pCKS07	C-V5-H6-*comW* in plasmid pEVP3	This study
pEVP3	Plasmid containing the Cm^r-encoding gene and the promoterless *lacZ* reporter derived from pTV32	7
pCR T7/CT TOPO	Plasmid for producing target proteins containing a C-terminal histidine tag	Invitrogen
pET16b	Plasmid for producing target proteins containing an N-terminal histidine tag	Novagen
pR410	Plasmid containing the *kan* gene	33
Primers		
CKS24	TATCTAGAGGCTGGTATTTTAACAATTCA	Upstream of *comW* (CP1721)
CKS25	ATGGATCCTCAACAAGAAATAAACCCCCGATTCA	In *comW* (CP1721)
CKS33	ATGTTACAAAAAATTTATGAGCAGATGGC	In *comW* (pCKS01)
CKS34	ACAAGAAATAAACCCCCGATTCATTACCAA	In *comW* (pCKS01)
CKS107	ATTCTAGAGCAGATGGCTAATTTCTATGATAGT	In *comW* (CP1802)
CKS108	ATGGATCCTCCGGATATAGTTCCTCCTTTCAG	In pCR T7/CT TOPO (CP1802)
CKS109	GGCATATGATTATGTTACAAAAAATTTATGAGC	In *comW* (pCKS02)
CKS110	ATGGATCCTCAACAAGAAATAAACCCCCGATTCA	In *comW* (pCKS02)
CKS116	GATCTAGAAAGGAGATATACCATGGGCCATCA	In pET16b (CP1805)
CKS114	ATGGGCCCTAGAAGTCTCAGCGAGCTCCATT	Upstream of *comW* (CP1805)
CKS115	GCTCTAGATTATAAACTTATTCTAACAAAAAA	Upstream of *comW* (CP1805)
DAM303	AAGGGCCCGTTTGATTTTTAATG	In pR410 (KANT)
DAM304	AGGATCCATCGATACAAATTCCTC	In pR410 (KANT)
DAM497	CAATTGACTATATTAGAGGCGAGACA	Upstream of *comW* (CP1805)
CKS113	ATGGATCCTTCTTCTTAGAAAAGGCCGTTTTA	Upstream of *comW* (CP1805)

ice, centrifuged at 10,000 rpm for 10 min at 4°C, and resuspended in 0.1 ml of gel loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, and 10% glycerol) (30). After being heated at 95°C for 5 min, each extract was subjected to SDS gel eletrophoresis, using either 12% Tris-glycine SDS gels with 4% stacking or 4 to 20% gradient Ready Gel Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA). Electrophoresis at 15 V/cm in Tris-glycine buffer was followed by transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) for 75 min at 46 V in transfer buffer (48 mM Tris, 39 mM glycine, pH 9.2). This membrane was blocked overnight at 4°C in TBST buffer (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk solids (Bio-Rad, Hercules, CA) and then probed with a purified polyclonal antibody against σ^X (20) at a dilution of 1:2,000 in TBST containing 1% nonfat milk solids for 2 h at room temperature. After being washed in 30 ml of TBST five times at room temperature, the membrane was incubated with a secondary peroxidase-conjugated anti-rabbit immunoglobulin G antibody (1:20,000 dilution; Amersham Biosciences, Piscataway, NJ) in TBST containing 1% nonfat milk solids for 1 h at room temperature and washed in TBST five times. The signal was detected by using ECL-Plus Western blotting reagents (Amersham Biosciences) and X-OMAT Imaging films (Kodak, Rochester, NY). The films were scanned using a ScanMaker 4850 (Microtek International Inc.) at 600-dot/in resolution, and band intensities were then compared with standards included in the same gels by using AlphaEase FC (Alpha Innotech, San Leandro, CA) for quantification.

Competence assay. Wild-type (WT) strain CP1250 and mutant pneumococcal strains were grown in CAT containing 8 mM HCl, 0.2% BSA, and 0.5 mM $CaCl_2$. At an OD_{550} of 0.05, each culture was split in half, and one half was induced by addition of CSP (200 ng ml^{-1}). One hundred ng ml^{-1} of Nov^r donor DNA (from strain CP1500) was added to both induced and uninduced cultures, and then each sample was incubated at 37°C for 1 h. After serial dilution, 0.1 ml of cells and 1.5 ml of CAT were mixed with 1.5 ml of melted CAT agar and poured onto a 3-ml-thick layer of CAT agar in a 60-mm-diameter petri dish. The plate was then covered with a 3-ml layer of CAT agar and another layer of CAT agar containing 30 μg Nov. Nov^r transformants were counted after 20 h at 37°C. For the experiment depicted in Fig. 1, cells were grown at 37°C in CAT supplemented with 8 mM HCl. At an OD_{550} of 0.05, 1 ml of each culture was added to 9 ml of warm CAT containing 8 mM HCl, 0.5 mM $CaCl_2$, and 0.2% BSA, and 1 ml of the dilution was transferred to a warm Eppendorf tube. After 20 min at 37°C, CSP was added to 200 ng ml^{-1}. At the indicated times, 0.1-ml culture samples were mixed with 10 ng of Nov^r DNA, incubated for 5 min at 37°C, and then diluted 1:150 into 1.5 ml of CAT containing 7.5 μg DNase. After 60 min at 37°C, the entire culture was mixed with 1.5 ml of melted CAT agar and plated as described above. Nov^r transformants were counted after 20 h at 37°C.

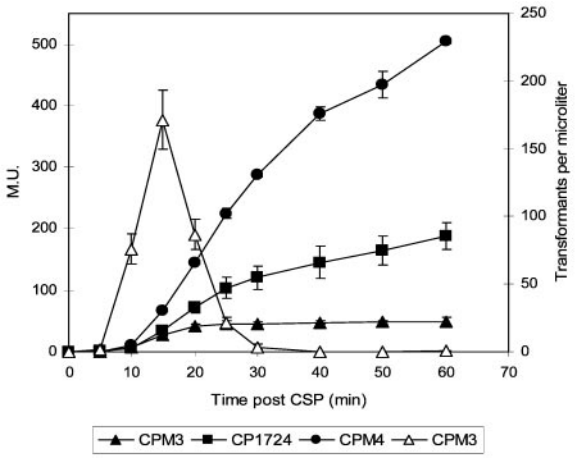


FIG. 1. Effect of *comW* mutation on the transcription of *comX*. Beta-galactosidase activities in CPM3 (*comX lacZ*; WT), CPM4 (*comX lacZ*; Δ*comX*), and CP1724 (*comX lacZ*; Δ*comW*) were assayed at the indicated times after CSP treatment. Competence of CPM3 is also shown (△). Transformation of CPM4 and CP1724 yielded fewer than 100 Nov^r/ml. Error bars, standard deviation among three replicate experiments. M.U., Miller units.

TABLE 3. CSP induction of *lacZ* fusions to *comW*, *ssbB*, *celB*, and *cglA* genes in different genetic backgrounds

Strain and relevant genotype	*lacZ* fusion site[a]	Beta-galactosidase activity[b]	
		W/o CSP	CSP
CP1721 WT	*comW*	0.5 ± 1	140 ± 100
CP1731 *comE*	*comW*	1 ± 1	0.5 ± 1
CP1723 *comX*	*comW*	0.5 ± 1	450 ± 250
CPM7 WT	*ssbB*	0	80 ± 40
CP1714 *comW*	*ssbB*	0.5 ± 1	10 ± 1
CP1601 *celB*	*celB*	1 ± 1	60 ± 40
CP1719 *celB comW*	*celB*	2 ± 2	2 ± 1
CP1822 *celB comW clpP*	*celB*	1 ± 1	5 ± 5
CP1823 *celB clpP*	*celB*	2 ± 1	30 ± 20
CP1548 *cglA*	*cglA*	0	300 ± 300
CP1718 *cglA comW*	*cglA*	1 ± 1	20 ± 10
CP1820 *cglA comW clpP*	*cglA*	1 ± 1	20 ± 20
CP1821 *cglA clpP*	*cglA*	2 ± 0	150 ± 100

[a] *lacZ* reporter gene was inserted into indicated locus.

[b] In each of three experiments, cells were grown in complete CAT medium supplemented with 8 mM HCl to an OD_{550} of 0.05, and a noncompetent culture and a parallel sample of the same culture treated with CSP for 20 min were prepared. Both samples were lysed and assayed for β-galactosidase as described in Materials and Methods. The average activity (Miller units) for three experiments is also shown, with standard deviations (SD) or range. W/o, without.

RESULTS

***comW* is required for the induction of late competence genes.** The temporal transcription pattern of *comW* is typical of early CSP-induced genes that are dependent on ComE but not on σ^X for their CSP responses, and its induction is not blocked in *comX* mutants (27). To determine more directly whether the competence regulators ComE and σ^X are required for the induction of *comW*, a *comW-lacZ* fusion reporter was assayed in an isogenic wild-type (CP1721), *comE* mutant (CP1731), or *comX* mutant (CP1723) background. Consistent with the known temporal expression pattern of *comW*, the induction of this *comW* transcriptional fusion depended on the competence regulator ComE, but not on the alternative sigma factor σ^X (Table 3). Indeed, CSP-induced transcription of the *comW* reporter was higher in the *comX* background than in the WT, as is typical of other early genes (16, 27). Previous work (2, 27) showed that *comW* is essential for competence for genetic transformation, and here we show that this gene is under the control of ComE and is independent of σ^X for its induction by CSP. It therefore seems possible that the deficiency of competence in *comW* mutants might be caused by reduced expression of late competence genes that are downstream of *comX* and are required for the processes of DNA uptake or recombination. To ask whether there was such an effect on late gene expression, the *lacZ* reporter was fused to the late competence genes, *celB*, *cglA*, and *ssbB*, which are required for genetic transformation, in either wild-type or *comW*-deficient backgrounds. The CSP-dependent induction of all three late competence genes was reduced in the *comW* background by at least 90% compared to that of the same late genes in the wild type (Table 3). This shows that *comW* precedes at least these three late genes in the CSP-induced signal cascade and is essential for maximal induction of DNA machinery genes. This result also suggests that the competence deficiency of a *comW* mutant may be due to reduction in the levels of DNA-processing machinery components.

Transcription of *comX* is strongly induced in a *comW* mutant. Since several σ^X-dependent late genes were only weakly induced by CSP in the *comW* background, it seemed possible that the expression of *comX* itself might be affected by the *comW* deletion. To test this hypothesis, a *comX-lacZ* transcriptional fusion was examined in either a wild-type (CPM3) or *comW* (CP1724) background in comparison to the *comX*-deficient *comX-lacZ* fusion strain CPM4, which was included in the study to reflect the strong negative autoregulatory effect of σ^X. The transcriptional pattern of *comX* in the *comW* mutant was very similar to that in the *comX* double mutant: both were higher than that seen in the wild-type background, and both continued well beyond 25 min (Fig. 1). Since the *comX-lacZ* fusion in both CP1724 and CPM3 has a native *comX* promoter, this result indicates that ComW is not needed for the strong induction of transcription of *comX* upon CSP treatment.

The rapid shutoff of expression of early competence genes, including *comCDE*, *comX*, and *comW*, appears to depend on σ^X, since in its absence, the transcription of early genes induced by CSP does not promptly return to the initial basal level, like that of the same genes in a wild-type background, but continues for at least two generations (16, 27). As shown in Fig. 1, beta-galactosidase activity was higher in the *comW* mutant (CP1724; *comX-lacZ* Δ*comW*) than in the *comW*$^+$ isogenic control (CPM3; *comX-lacZ*). The activity of beta-galactosidase also rose continuously for at least 60 min in CP1724, while the activity in the wild-type background leveled off after 20 min. Therefore, the negative autoregulatory mechanism that is proposed to be mediated by *comX* or a *comX*-dependent late gene(s) is similarly dependent on *comW*.

The amount of the σ^X protein is very small in *comW* mutants. Since in *comW* mutants there was a high level of *comX* transcription but a very low transformation rate and low expression of *comX*-dependent genes, indicating an apparent strong reduction of σ^X activity, we sought to determine the actual level of σ^X in *comW* mutants directly. Western blotting analysis was performed in the *comW* mutant CP1376 and its parent, CP1250, using an antibody against σ^X. One might expect to see an elevated level of σ^X in CP1376, since the *comX-lacZ* transcriptional fusion in strain CP1724 had such a high expression level. However, in contrast to the pattern observed for the transcriptional reporter, the level of σ^X found in the *comW* mutant was low, although it did appear transiently, with kinetics similar to those of the wild type and with a maximum at 15 min (Fig. 2A and B). The maximal σ^X level was ~10% of that seen in the wild-type strain (Fig. 3 and Table 4). Therefore, ComW appears to act after initiation of transcription of *comX* and to affect the level of σ^X protein that accumulates. Thus, ComW may act during translation of *comX* (e.g., by stabilization of mRNA or initiation of translation) or for the stabilization of σ^X after its synthesis.

ComW is required for protection of σ^X from proteolysis. The ATP-dependent ClpP protease is responsible for degradation of many regulatory proteins in both gram-positive and -negative bacteria (35, 38, 39), as well as for removal of misfolded proteins (14). In *S. pneumoniae*, *clpP*-deficient mutant strains can exhibit a precocious and extended period of endogenous competence (29), which is partly explained by an ele-

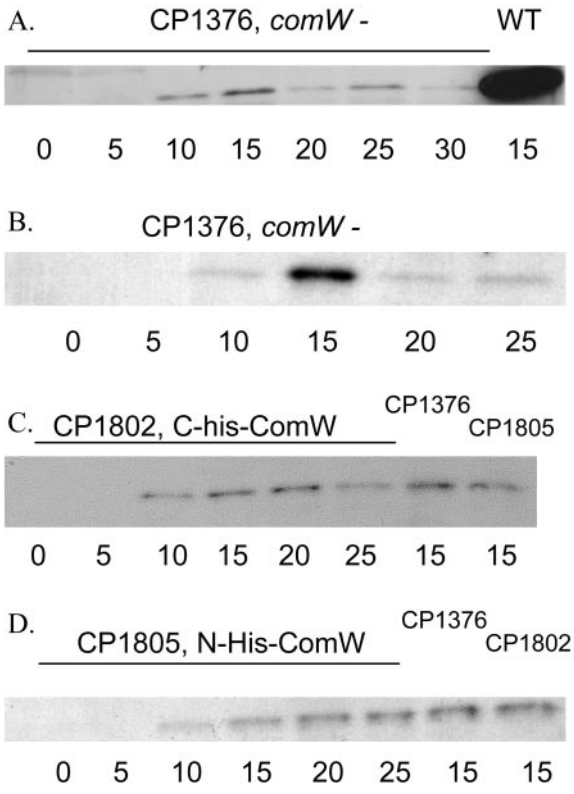


FIG. 2. Accumulation of the σ^X protein requires ComW. The WT (CP1250) and a *comW* mutant (CP1376), a C-His-tagged ComW mutant (CP1802; 0- to 25-min samples in panel C), and an N-His-tagged ComW mutant (CP1805; 0- to 25-min samples in panel D) were treated with CSP (at time zero), and samples were collected as described in Materials and Methods at the indicated times (in minutes). An extract representing 1 ml of culture was loaded into each well. Samples of CP1376 from two independent preparations were loaded onto different gels (A and B). The gels were assayed by Western blotting using an antibody against σ^X.

vated basal expression level of the *comC* gene encoding the competence pheromone (6). It has also been reported that mutation of the *clpP* genes in *S. pneumoniae* and *S. pyogenes* leads to increased accumulation of σ^X, suggesting that ClpP protease may negatively control σ^X in both of these streptococci (17, 23). As σ^X is more stable in a *clpP* mutant, and as its accumulation depended on the ComW protein (Fig. 2), we created a *clpP*::Tet *comW*::KANT double mutant to distinguish whether ComW is involved in translation of *comX* or in the stability of σ^X after translation. If the synthesis of σ^X depends on ComW, the amount of σ^X produced in response to CSP treatment would be low in this *clpP comW* double mutant. However, σ^X would accumulate to high levels after CSP induction in the double mutant if ComW acts later, by preventing ClpP-proteolysis of σ^X, as the *clpP* mutation would replace the missing protective function.

In the double mutant CP1815, the level of σ^X at 15 min postinduction was in fact higher (threefold) than that seen at 15 min in the wild type and approximately twice that in the *clpP* single mutant (Fig. 3 and 4 and Table 4), indicating that in this strain, production of σ^X does not depend on ComW. Therefore, translation of *comX* is independent of ComW, and the role of ComW in determining the level of σ^X appears to be protection of the protein from proteolysis by ClpP. The immunoblotting results also indicate that σ^X accumulated to a higher level (fivefold by 80 min) (Fig. 3) in CP1815 (*clpP comW*) than in the *clpP* single mutant CP1359. This greater accumulation of σ^X in CP1815 may be explained by increased and prolonged transcription of the *comX* gene in the *comW*-deficient mutant strain due to reduction of the σ^X-dependent shutoff processes, as indicated in Fig. 1.

ComW is also required for full activity of σ^X. To ask whether absence of the σ^X-degrading ClpP protease rescues the deficiency of competence found in the *comW* single-mutant strain, a *clpP comW* double mutant, CP1815, was also examined for its competence. Surprisingly, the competence of CP1815 was <0.1% of normal (Table 5), even though this mutant had a normal amount of σ^X after CSP treatment (Fig. 3C and 4C). Thus, ComW is still required for genetic transformation in the *clpP*-deficient background. To ask whether the competence deficiency of the *clpP comW* double mutant could reflect a deficiency in expression of late genes that are essential for the DNA uptake process and recombination, late *lacZ* reporter fusions were examined in *clpP* single- or *clpP comW* double-mutant backgrounds. In the *clpP comW* double mutant, transcription of the late genes, *celB* and *cglA*, was ~10% of that seen in CSP-treated *clpP* single mutants (Table 3). As the quantitative relationship between competence and late-gene expression is not known in detail, and as only two late genes were sampled, it is not clear whether the 90% decrease in transcription of *celB* and *cglA* fully explains the 99.9% decrease in competence. Nevertheless, we conclude that the deficiency of competence in the *clpP comW* strain was due, at least in part, to reduced expression of DNA machinery genes, possibly caused in turn by low activity of the abundant σ^X. Thus, ComW appears to have another important function in genetic transformation, a function needed for full activity of the competence-specific σ. It is possible that this other function of ComW could be to modify σ^X to an active form, to release it from sequestration, or to cooperate with it in transcription.

A *clpE* mutant still requires *comW* for genetic transformation. The ClpP proteolytic subunit is known to associate with several different ATP-binding subunits to recognize and degrade different substrates (9). In *S. pneumoniae*, the putative ATP-binding subunits of Clp protease include ClpC, ClpE, ClpL, and ClpX (34). Luo recently found that σ^X remains stable after competence induction in *clpE* or *clpP* (but not in *clpC* or *clpL*) backgrounds, suggesting that ClpE may associate with the ClpP proteolytic domain for proteolytic regulation of σ^X (17). To explore the possibility that the inactive state of σ^X in the *clpP comW* mutant was caused by ClpE-mediated sequestration, a *clpE* deletion mutation was introduced into this double mutant, creating the *clpP clpE comW* mutant, CP1852, and the competence of this triple mutant was determined. The transformation rate of the *clpP clpE comW* mutant was the same as that of the *clpP comW* parent (<0.1% of that seen in the wild-type strain) (Table 5). Thus, the activity of σ^X in a *clpP comW* mutant was not increased by an additional *clpE* mutation, and its low apparent activity in the *clpP comW* genetic background must not be due to sequestration by ClpE.

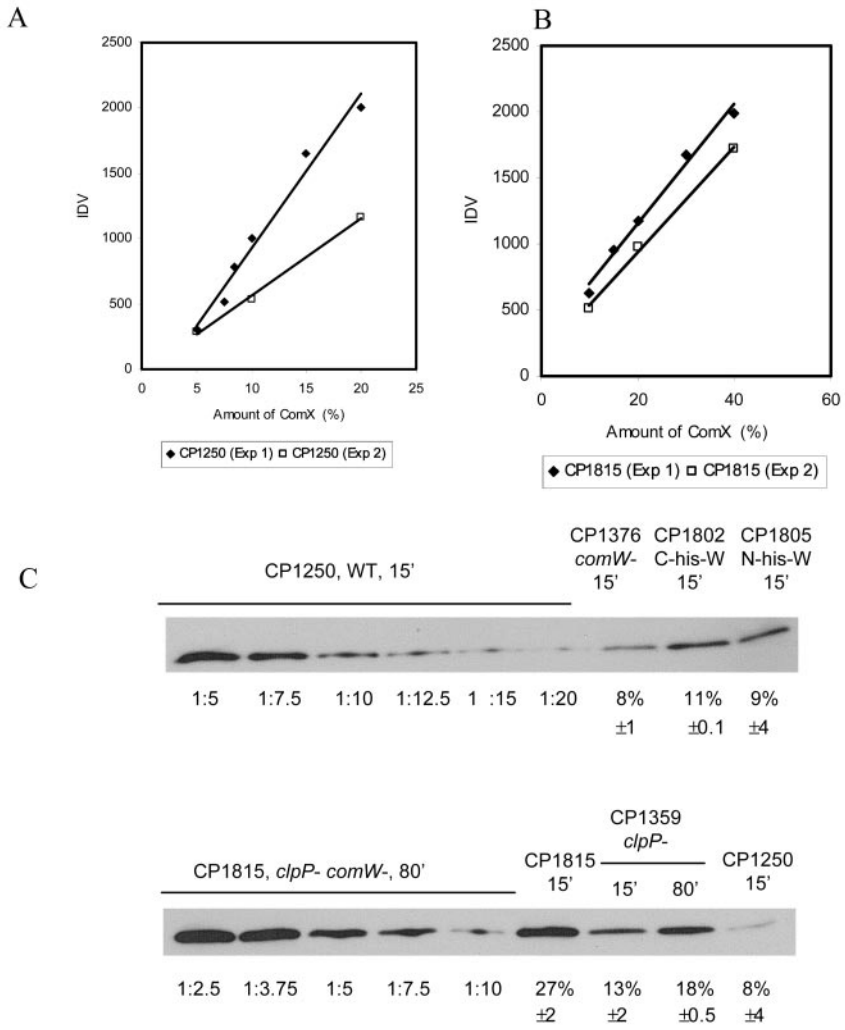


FIG. 3. Quantification of σ^X in various genetic backgrounds. (A) σ^X standard curve prepared from a 15-min extract of CP1250 (WT). A sample (100%) was prepared from CP1250 at 15 min post-CSP treatment, and then different amounts (dilutions of 1:5, 1:7.5, 1:10, 1:12.5, 1:15, or 1:20) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. The relationship between the integrated density values (IDV) of enhanced chemiluminescence assay bands and relative amounts of protein is shown. (B) σ^X standard curve prepared from an 80-min extract from CP1815 (*clpP comW* double mutant). The extract was serially diluted in a blank extract of CPM8 (Δ*comX*) (dilutions of 1:2.5, 1:3.75, 1:5, 1:7.5, or 1:10) before assay by SDS-PAGE and Western blotting. (C) Immunoblotting image of σ^X in a wild-type strain and various mutant strains. The relative amount of σ^X in each strain is shown, with a range. The intensities of CP1376, CP1802, and CP1805 were compared to the CP1250 standard curve (A), while those of CP1250, CP1815, and CP1359 were compared to the CP1815 standard curve (B).

Antiprotease activity is genetically distinguishable from the activation function of ComW. N- or C-terminal portions of many bacterial regulators fulfill separate roles or target different molecules (21, 22, 24, 32). Since the ComW protein appears to have dual functions, both protecting and activating σ^X, it is possible that its termini might also be important for different roles. To explore this possibility, mutant strains having C- or N-terminal histidine modifications of σ^X (Fig. 5) were constructed and examined by immunoblotting and a competence assay. The levels of σ^X in cells expressing N- or C-tagged ComW forms were low, similar to that seen in the *comW* deletion mutant CP1376 (Fig. 4C and Table 5). Thus, both ends of this protein are important for the stabilization or protection of σ^X. However, the levels of competence of the two mutant strains differed. Competence was <0.1% of the control in the strain expressing an N-His-tagged ComW, just as for a *comW* deletion mutant (Table 5). In contrast, the competence of the strain expressing a C-His-tagged ComW was 70% of the normal level (Table 5), even though the amount of σ^X in this strain after CSP induction was 11% of that in a wild-type strain (Fig. 3C and Table 4). Therefore, the amino terminus is important for the activity of σ^X, but both amino and carboxyl termini of ComW are important for promoting its stability.

DISCUSSION

The alternative sigma factor σ^X is known to provide a link between the early quorum-sensing signal and the expression of late DNA machinery genes during development of competence in *S. pneumoniae*, and here we report evidence that another

TABLE 4. Quantification of ComX in various pneumococcal strains

Protein sample and time[a]	IDV[b]	Avg relative amt (%) ± range[c]
CP1250 (WT) 15		100
CP1376 (Δ*comW*) 15	600 ± 180	8 ± 1
CP1802 (C-his-ComW) 15	800 ± 200	11 ± 0.1
CP1805 (N-his-ComW) 15	750 ± 400	9 ± 4
CP1815 (Δ*clpP*, Δ*comW*) 80		100
CP1815 (Δ*clpP*, Δ*comW*) 15	1,300 ± 170	27 ± 2
CP1359 (Δ*clpP*) 15	750 ± 100	13 ± 2
CP1359 (Δ*clpP*) 80	1,000 ± 100	18 ± 0.5
CP1250 (WT) 15	550 ± 50	8 ± 4

[a] Samples were prepared at indicated times (in min) post-CSP treatment.
[b] The intensity of each band was converted into an IDV using Alpha-Ease FC.
[c] The average of the relative amounts of ComX determined by two independent experiments is shown, with a range. The relative amount of the ComX protein was determined by the IDV of each sample and standard curve graphs. (The graph of serially diluted samples of CP1250 at 15 min [Fig 3A] was used for CP1376, CP1802, and CP1805; the graph of serially diluted samples of CP1815 at 80 min [Fig 3B] was used for CP1815 at 15 min, CP1359 at 15 min, CP1359 at 80 min, and CP1250 at 15 min.)

early competence gene product, ComW, also plays important roles in that linkage through the regulation of *comX*.

Regulation of a number of proteins, including alternative sigma factors, by proteolysis has been observed in a wide variety of bacterial species (reviewed in reference 13). Among well-known proteases, ClpP is an important regulatory factor. ClpP degrades sigma factor S in nonstress growth conditions in *E. coli* (31), in a proteolytic process that depends on a targeting factor, RssB, for delivery of sigma S to the ClpXP protease (39). ClpP also controls the development of competence in *B. subtilis* by degrading a competence-specific activator, ComK (35). *B. subtilis* does not possess a secondary sigma factor linking quorum sensing and DNA machinery gene expression, but the release of the transcriptional activator ComK from the ClpCP/MecA complex by ComS leads to the development of competence (36). In *S. pneumoniae*, endogenous competence induction in a *clpP*-deficient mutant strain can extend longer than in the WT, suggesting that ClpP may also, as in *B. subtilis*, be a negative regulator of competence induction (29). Chastanet et al. showed that deletion of the *clpP* gene caused an elevated basal expression level of the *comC* gene encoding the pneumococcal competence pheromone (6). Mutation of the *clpP* gene also leads to an increased accumulation of the alternative sigma factor σ^X, indicating that the ClpP protease negatively affects σ^X levels in this species (17). The induction of ComW by the quorum-sensing circuit seems to prevent this proteolytic process temporarily, so as to amplify the effect of *comX* induction in coordinating competence development. In the wild-type pneumoccocal cell, σ^X disappears after activating the DNA machinery genes by a ClpP-mediated proteolytic process. This suggests that ComW may protect σ^X only briefly, at the start of competence. The basis of this temporary protection is not known, but possibilities include that ComW itself may be very unstable, perhaps having strong affinity to a Clp protease, or an initial ComW-σ^X complex may be dissociated by a late competence protein, allowing the Clp protease access to σ^X.

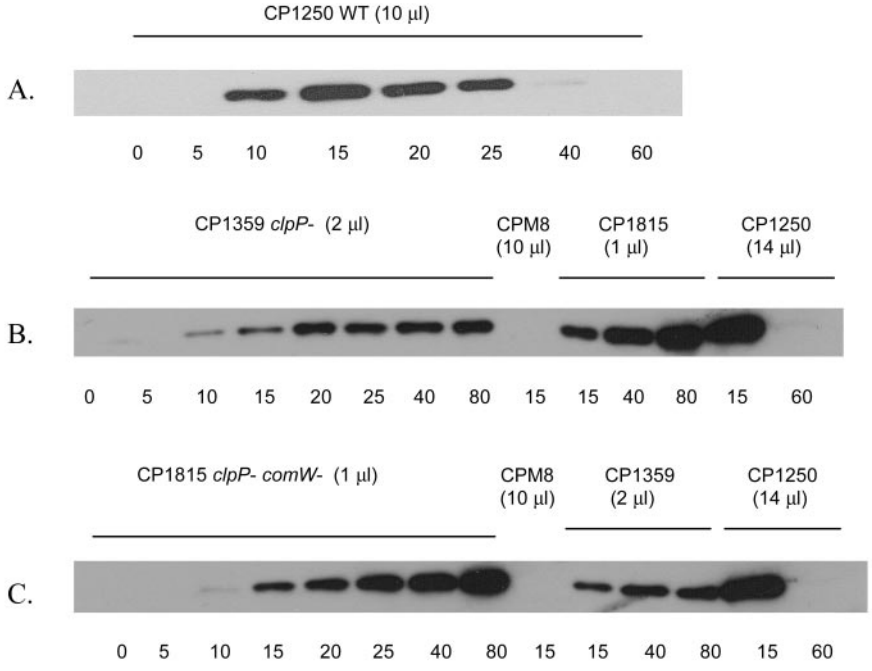


FIG. 4. Deletion of both *comW* and *clpP* leads to the accumulation of σ^X after competence induction. The wild-type (CP1250; 0- to 60-min samples in panel A), a *comX* mutant (CPM8), a *clpP* single mutant (CP1359; 0- to -80-min samples in panel B), and a *comW clpP* double mutant (CP1815; 0- to 80-min samples in panel C) were induced by CSP (at time zero), and samples were withdrawn at the indicated times (in min) after induction. The indicated amount of each extract (0.1 ml of original culture per μl extract) were assayed by Western blotting analysis and probed with anti-ComX antibody.

TABLE 5. Competence for transformation in WT and *comW*, *clpE*, and *clpP* mutants of *S. pneumoniae*

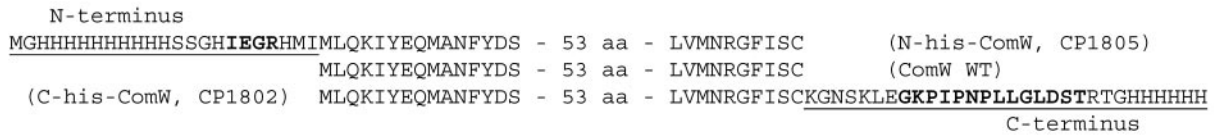
Strain	Description	Transformants/ml (± SD or range)[a]		Relative competence[b] (%) (± SD or range)
		W/o CSP	CSP	
CP1250	WT	<0.01	680 ± 310	100
CP1376	*comW*	<0.01	<0.01	<0.01
CP1802	C-V5-H6-ComW	<0.01	440 ± 170	80 (±10)
CP1805	N-H6-Xa-ComW	<0.01	<0.1	<0.1
CP1359	*clpP*	<0.01	530 ± 350	80 (±15)
CP1815	*clpP comW*	<0.01	0.3 ± 0.1	<0.05
CP1816	*clpP* N-H6-Xa-ComW	<0.01	0.3 ± 0.1	<0.05
CP1850	*clpP* C-V5-H6-ComW	<0.01	270 ± 35	70 (±10)
CP1851	*clpE*	<0.01	720 ± 480	90 (±30)
CP1852	*clpP clpE comW*	<0.01	0.2 ± 0.0	<0.05
CP1853	*clpP clpE*	<0.01	1,100 ± 120	110 (±10)
CP1854	*clpE comW*	<0.01	<0.01	<0.001

[a] The average number of transformants determined by two or three independent experiments is shown, with a range or standard deviation. CP1500 Nov^r donor DNA was used in each assay. W/o, without.
[b] Transformant yield compared to CSP-induced WT (CP1250) in parallel control cultures.

ComW is also required for activity of σ^X by an unknown mechanism. If σ^X is initially produced as an inactive form that needs to be processed to cooperate with RNA polymerase, ComW may participate in its processing to an active form. Another possibility is that ComW may release σ^X from sequestration. The ATP-dependent Clp protease subunits are known to recognize substrates in a specific manner, allowing access to the proteolytic subunit, ClpP. Thus, candidates for sequestration of σ^X include the ATP-binding subunits ClpC, ClpE, ClpL, and ClpX. A genetic test of the most likely of these candidates, *clpE*, indicated that it is not responsible for the inactivity of σ^X in the *clpP comW* background, but other factors may cause it to be inactive in the absence of ComW. It is also possible that ComW may cooperate directly with σ^X or RNA polymerase in the initiation of transcription to promote the interaction or combined functions of these two molecules.

CSP-induced mRNA levels for early competence genes, including *comCDE*, *comX*, and *comW*, reach a maximum at ~7.5 min and then sharply decline. This decay depends on *comX*, since the induced transcription of these genes persists much longer in a *comX*-deficient background (16, 27). In a *comW* mutant (CP1724), the transcriptional expression pattern of *comX* was initially similar to that observed in a WT strain (CP1250). After 15 min, however, a significant amount of *comX* mRNA continued to be made in CP1724 for at least 60 min, while no message was evident in the WT after 25 min, presumably due to the putative competence repressor(s) (Fig. 1). Since this expression pattern indicates that the *comW* mutant is deficient in competence (or early-gene) shutoff, the negative regulatory mechanism appears to be dependent on *comW*. Figure 1 also shows that the accumulation of beta-galactosidase in CP1724 slows slightly after 20 min. This suggests that the shutoff mechanism for early genes may be partially functional in the *comW* background. As a *comW* mutant accumulates ~10% of the normal σ^X level after induction and achieves 5 to 10% of the normal levels of expression of several late genes, this partial shutoff may be explained by such residual activity of σ^X.

N- or C-terminal domains of many bacterial regulators have separate roles, such as targeting different molecules (21, 22, 24, 32). In *Bacillus subtilis*, MecA, which acts as an adapter protein targeting the competence transcription activator ComK for Clp degradation (35, 36), appears to bind two different molecules, ComK and ClpC, at its N- and C-terminal domains, respec-



Strain	Competence	σ^X protein
CP1250 (WT)	100	100
CP1802 (C-his-ComW)	80 +/- 10	11 +/- 0.1
CP1805 (N-his-ComW)	<0.1	9 +/- 4
CP1376 (Δ*comW*)	<0.1	8 +/- 1

FIG. 5. Effects of terminal extensions of the ComW protein on competence and σ^X accumulation. The sequences of WT ComW (middle), amino-terminally His-tagged ComW (CP1805; top), and carboxyl-terminally His-tagged ComW (CP1802; bottom). His tags introduced into the wild-type ComW are underlined; boldface residues in N-His-ComW indicate a factor Xa cleavage site; boldface residues in C-His-ComW indicate a V5 epitope. Omitted 53 residues: IEEEYGPTFGDNFDWEHVHFKFLIYYLVRYGIGCRKDFIVYHYRVAYRLYLEK. The corresponding competence phenotype (mean relative number of transformants ± standard deviation) and relative amount of σ^X (mean and range) observed 15 min after CSP induction are also shown. aa, amino acids.

tively. The amino-terminal domain of MecA binds to transcription factor ComK, while the carboxy-terminal domain interacts with an ATP-binding subunit, ClpC. These interactions cause the formation of a ternary complex, leading to degradation of the ComK protein by the ClpP proteolytic subunit (24, 35, 36). The results presented here indicate that the N and C termini of ComW may also have different targets. Both the C and N termini of the ComW protein are required for the protection of σ^X from proteolysis, but only its amino terminus appears to be important for its activation. However, functionally important portions of this protein could be mapped more precisely in the future by use of mutant proteins.

While alternative sigma factors are powerful regulatory devices, coordinating global regulation, they have drawbacks. Transcription read-through into such genes might lead to initiation of a signal pathway at the wrong time, while mutations affecting expression or function of these cellular regulators may easily have serious pleiotropic effects. Thus, many bacteria possess additional factors that help to tighten the regulation of alternative sigma factor activity. In the case of *S. pneumoniae* competence, σ^X is subjected to primary transcriptional control by ComE and the quorum-sensing circuit, but a more secure regulation of genetic competence may be achieved by means of an additional regulatory factor, ComW, which is required for both stabilization and activation of this alternative σ.

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