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Choline-Binding Protein D (CbpD) in *Streptococcus pneumoniae* Is Essential for Competence-Induced Cell Lysis

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Streptococcus pneumoniae **is an important human pathogen that is able to take up naked DNA from the environment by a quorum-sensing-regulated process called natural genetic transformation. This property enables members of this bacterial species to efficiently acquire new properties that may increase their ability to survive and multiply in the human host. We have previously reported that induction of the competent state in a liquid culture of *Streptococcus pneumoniae* triggers lysis of a subfraction of the bacterial population resulting in release of DNA. We have also proposed that such competence-induced DNA release is an integral part of natural genetic transformation that has evolved to increase the efficiency of gene transfer between pneumococci. In the present work, we have further elucidated the mechanism behind competence-induced cell lysis by identifying a putative murein hydrolase, choline-binding protein D (CbpD), as a key component of this process. By using real-time PCR to estimate the amount of extracellular DNA in competent relative to noncompetent cultures, we were able to show that competence-induced cell lysis and DNA release are strongly attenuated in a *cbpD* mutant. Ectopic expression of CbpD in the presence or absence of other competence proteins revealed that CbpD is essentially unable to cause cell lysis on its own but depends on at least one additional protein expressed during competence.**

Streptococcus pneumoniae and related streptococci have the ability to take up exogenous DNA for potential genomic transformation. Mapping has shown that this intrinsic property is not constitutive but regulated by the competence-stimulating peptide (CSP) and its cognate signal transduction pathway, ComDEX (13, 14, 17, 25). ComD, which is a transmembrane histidine kinase, monitors the concentration of CSP outside the cytoplasmic membrane. At the critical concentration of CSP, which is affected by the growth conditions used, the competent state is turned on (7).

Recent mapping of CSP-responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays has shown that eight early genes and 14 late genes are required for transformation (26). The seven proteins encoded by the essential early genes include the CSP secretion apparatus (ComAB), the precursor of CSP (ComC), the two-component regulatory system ComDE, the alternative sigma factor ComX (encoded by two identical genes, *comX1* and *comX2*), and the newly discovered positive regulator of competence termed ComW (18). The 14 essential proteins encoded by the late genes are members of the ComX regulon and include DNA-processing proteins plus the components of the DNA uptake machinery. Interestingly, the 22 genes that are indispensable for transformation constitute only about 18% of the CSP-inducible genes in *Streptococcus pneumonia* (26). This finding immediately raises the question of whether CSP could regulate other biological functions in addition to natural transformation.

Several studies have reported that ComD mutants are attenuated with respect to pathogenicity, indicating that at least one of the CSP-responsive genes contributes to virulence (12, 16, 19). It is also possible that some of the CSP-responsive genes are involved in other aspects of horizontal gene transfer than uptake and integration of incoming DNA. We have previously shown that a subfraction of the cell population lyse when a liquid culture of pneumococci is induced to competence by addition of CSP (35, 36). The biological significance of this phenomenon is not clear, but an attractive hypothesis is that some cells lyse to release DNA that can be taken up by the others. The molecular mechanism behind the observed cell lysis remains to be elucidated, but previous studies strongly indicate that choline-binding proteins are involved (35). Choline-binding proteins contain a number of short choline-binding repeats which anchor them noncovalently to teichoic and lipoteichoic acid in the pneumococcal cell wall (9, 33). Here we show that CbpD, a CSP-inducible choline-binding protein and putative murein hydrolase, plays a key role in competence-induced cell lysis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study were derived from *S. pneumoniae* strain Rx (see Table 1). Bacterial strains were grown in CAT medium or on CAT agar plates containing (per liter) 167 mmol of K_2HPO_4, 5 mg of choline chloride, 5 g of tryptone, 10 g of enzymatic casein hydrolysate, 1 g of yeast extract, and 5 g of NaCl (22). After sterilization, glucose was added to a concentration of 0.2%.

Construction of *S. pneumoniae* mutants. To investigate the expression profile of *cbpD* during competence induction, the *S. pneumoniae* L1 mutant strain was constructed (Table 1). First, a 400-bp internal fragment of *cbpD* was amplified from the CP1415 strain using the primers cbpDrapFor (5′-GAGAGAGCATG CGGAAGGTTATCGTGTAGATAATACACCG-3′) and cbpDrapBack (5′-CT CTCTGGATCCGGCAGTATAACTCAACCACTTATAGCCG-3′). Then the fragment was digested with BamHI and SphI and ligated into the corresponding restriction sites of the nonreplicative pEVP3 vector (6), resulting in a transcriptional fusion between the *cbpD* gene and the pEVP3 *lacZ*. The resulting vector

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TABLE 1. *S. pneumoniae* strains used in this study

Strain	Genotype and construction	Source or reference
Rx	R36A derivative	Ravin (29)
CP1200	Rx but *malM511 str-1*	Shoemaker and Guild (34)
CP1415	CP1200 but *comA* negative by insertion of the ermAM cassette into the ClaI site of *comA*	Morrison et al. (20)
CP1500	*hex nov-r1 bry-r str-1 ery-r1 ery-r2*	Cato and Guild (5)
EK100	CP1415 but *egb* negative by transformation with genomic DNA from R262	Steinmoen et al. (35)
EK4166	EK100 but *hirL*::*pEVP3* by transformation with plasmid DNA	Steinmoen et al. (35)
H1	CP1415 but Nov^r by transformation with CP1500 DNA	Steinmoen et al. (35)
H2	H1 but *lytA*::*pEVP3* by transformation with plasmid DNA	Steinmoen et al. (35)
H3	H1 but *lytA*::*pEVP3* by transformation with plasmid DNA	Steinmoen et al. (35)
H5	EK100 but *lytC*::*pEVP3* by transformation with plasmid DNA	Steinmoen et al. (36)
EK4168	EK4166 but Δ*cbpD* (Materials and Methods)	This study
L1	EK100 but *cbpD*::*pEVP3* by transformation with plasmid DNA	This study
L3	CP1415 but Δ*cbpD* (Materials and Methods)	This study
L5	L3 but *qsrA*::*cbpD* (see Materials and Methods for details)	This study

was subsequently used to transform the *S. pneumoniae* EK100 strain (Table 1) by natural transformation. A single transformant, termed L1, was isolated from an agar plate containing 2.5 μg/ml chloramphenicol. Correct integration of the recombinant vector was confirmed by employing PCR with flanking primers followed by DNA sequencing of the PCR product.

Strain EK4166 was used to construct the *cbpD* deletion mutant EK4168 (Table 1). A 953-bp DNA fragment corresponding to the region immediately upstream of *cbpD* was amplified using the primers cbpD.5 (5′-ATTAGACGTCCTGACA TCTTCAAGTAATAACTTGTTCCT-CTTGC-3′) and cbpD.1 (5′-ATTAGCT AGCGCTGCAGAAGGCTTGGTGGAGTCAC-3′) and ligated into pCR2.1-TOPO (Invitrogen) according to the manufacturer's recommendations. The fragment was excised from the pCR2.1TOPO vector with AatII and NheI and ligated into multiple cloning site I of the nonreplicative vector pFW13 (27). Next, a 766-bp fragment corresponding to the region downstream of *cbpD* was amplified using the primers cbpD.3 (5′-ATTACCATGGGGCTGGTACCAGGTCT CTGGTGAGTG-3′) and cbpD.4 (5′-ATTACATATGGCCACTCTCAAGGTC GCCCAGCTATGG-3′) and cloned into a pCR2.1TOPO vector. The fragment was excised from pCR2.1TOPO with NcoI and SpeI and cloned into multiple cloning site II of the pFW13 vector harboring the 953-bp fragment. Using this construct as a template and the oligonucleotides cbpD.1 and cbpD.4 as primers, a 3,300-bp fragment consisting of the Kan^r gene of pFW13 flanked by the two cloned fragments described above was amplified. This linear DNA fragment was then used to transform EK4166 by natural transformation. A transformant growing on agar plates containing 100 μg/ml kanamycin was isolated, and correct insertion of the Kan^r cassette was verified by PCR and DNA sequencing.

cbpD deletion mutant L3, used in the quantitative PCR experiments, was made by transforming strain CP1415 with the 3,300-bp PCR fragment described above. The L5 mutant was constructed in order to compare the amount of DNA release mediated by CbpD when this protein was expressed together with or separate from the other competence proteins. First, an 842-bp DNA fragment encompassing the inducible p1 promoter of the *qsrAB* operon plus the ≈200-bp BOX element immediately downstream of the p1 promoter (15) was amplified by PCR using the primers Box1 (5′-ATATAGGATCCTATACTCAATGAAAATCAA AGAGC-3′) and 4144bgal2 (5′-ATTAAATGCATCTCTGATGGACGTAATT TATGGCTAG-3′). This fragment was digested with BamHI and NsiI and ligated into the corresponding sites of the pEVP3 vector, giving rise to the vector pEVP3*qsr*I.

To obtain a p1 promoter that would respond equally well to induction by CSP-1 and BIP-1, we introduced two point mutations in the right repeat of this promoter. This was done as described by Knutsen et al. (15) for the construction of the OE4145 mutant strain. Then, a unique SpeI restriction site was introduced in pEVP3*qsr*I close to the 3′ end of the cloned 842-bp fragment, using the primers mutSpeIF (5′ CCCCAGCTTGAATTGATACA CTAGTGCTTTTATATAGGGAAAAGGTGG-3′) and mutSpeIB (5′-CCA CCTTTTCCCTATATAAAAGCACTAGTGTATCAATTCAAGCTGGGG-3′) according to the Quick Change PCR mutagenesis method (Stratagene). Next, the *cbpD* gene was amplified from genomic CP1415 DNA, using the primers cbpDBDraIII (5′CTCTCTCACAGCGTGCTATACTCGTTCTCCA TCGTTTACTC-3′) and cbpDFspeI (5′-AGAGAGACTAGTCGCTTTATA TTATTTTTTAAGGAGGAAGAATG-3′). This fragment was digested with DraIII and SpeI and ligated into the corresponding sites of the mutagenized pVP3*qsr*I vector described above. The resulting vector, pVP3*qsr*II, was transformed into the L3 strain by natural transformation. Correct chromosomal integration was verified by DNA sequencing.

Assay of β-galactosidase activity. Competence-induced expression from the *cbpD* promoter was monitored using the L1 mutant strain. An overnight culture of L1 was diluted in CAT medium to an OD_{550} of 0.05. The culture was grown at 37°C. At selected optical densities, 1-ml samples were collected and incubated at 30°C for 10 min before addition of 250 ng/ml CSP. The tubes were subsequently incubated at 30°C for 30 min and cells were lysed by addition of 0.1% (vol/vol) Triton X-100. Incubation was carried out at 30°C because competence-induced cell lysis is less effective at 37°C (36). Parallel samples containing uninduced cells were collected as described above; 200 μl cell lysate was added to 250 μl 5x Z buffer [5 mM $MgCl_2$, 250 mM β-mercaptoethanol, 50 mM KCl, 0.3 M $Na_2HPO_4 \cdot 7H_2O$, 0.2 M $NaH_2PO_4 \cdot H_2O$, 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG), pH 7.0] and 760 μl CAT medium, and subsequently incubated at 30°C for 1 h. The β-galactosidase reaction was stopped by addition of 500 μl 1 M Na_2CO_3, and hydrolysis of ONPG was recorded in a spectrophotometer at 420 nm. Enzyme activity was calculated according to the method of Miller (20). β-Galactosidase release assays were performed as described by Steinmoen et al. (35), except that competence induction was performed at 30°C.

Real-time PCR assays. Overnight cultures were diluted to an OD_{550} of 0.05 in CAT medium and grown at 37°C to an OD_{550} of 0.3. Then a second dilution to an OD_{550} of 0.1 was performed. When the cultures reached an OD_{550} of 0.3, 0.5 μg/ml of salmon sperm DNA and 15 pg/ml of the plasmid pLNO2HM1 (24) harboring a variant of the *hm1* gene (4) were added. The cultures were next transferred to 30°C and treated with either 250 ng/ml CSP-1, 250 ng/ml BIP-1, or kept untreated. After 30 min of incubation, 1.5-ml samples were collected in microtubes and centrifuged at 4°C for 2 min at 15,000 × g. Then the supernatants were sterile filtered (pore size of 0.2 μm) into cooled cryotubes and stored at −80°C. Real-time PCR was performed on the supernatant fractions in triplicate reactions, using specific oligonucleotides for the pneumolysin gene (*ply*) and the *hm1* gene (Table 2), and the TaqMan universal PCR master mix according to the manufacturer's instruction (User Bulletin 2, Applied Biosystems, Foster City, USA). Real-time PCR was performed with the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). Standard curves were

TABLE 2. Primers and probes used in TaqMan real-time PCR analysis[a]

Gene	Probe (5′→3′)	Forward primer (5′→3′)	Reverse primer (5′→3′)
ply	ACTCTTACTCGTGGTTTCC	GGCGCCAAGTCTATCTCAAGTT	CTTCAAAAGCAGCCTCTACTTCATC
hm1	CAGACACTGGACCCTGC	GGCGCACACCCAATGC	CCCCTCGGTTCTTGTCTATCCA

[a] All probes were synthesized with a 5′-labeled 6-carboxyfluorescein reporter and a 3′-labeled nonfluorescent quencher.

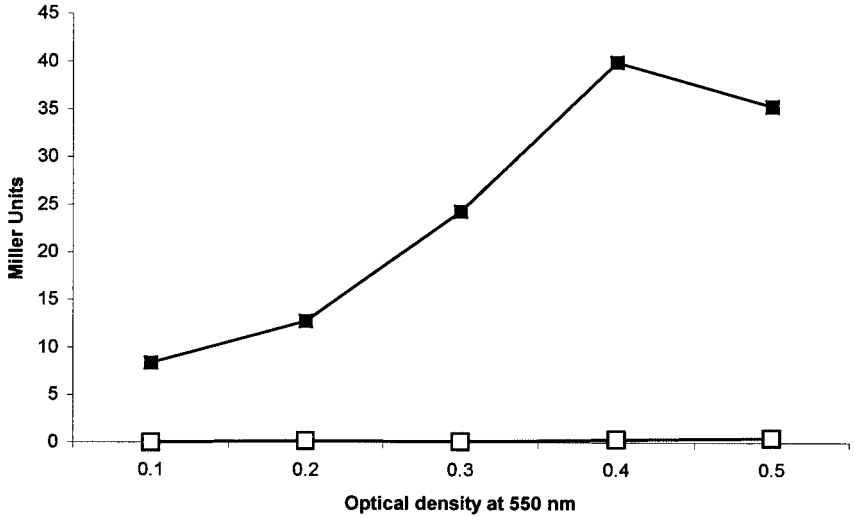

FIG. 1. *cbpD* promoter activity in competent and noncompetent cells. The L1 mutant was collected at various cell densities (OD_{550} of 0.1 to 0.5) and induced to competence by addition of 250 ng/ml of synthetic CSP-1. After 30 min at 30°C, cultures were lysed and assayed for β-galactosidase activity. Corresponding uninduced samples were run in parallel. Solid squares, cell lysates from cells induced with CSP-1; open squares, cell lysates from uninduced cells. The data presented are representative of results from three independent experiments. Strain L1 is *comA egb cbpD*::*pEVP3*.

obtained for both *ply* and *hm1* to verify that the amplification efficiency was similar in order to apply the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems) for the analysis. Plasmid DNA (*hm1*) was used as an internal control for data normalization. The amount of released DNA was expressed as the *n*-fold difference relative to the untreated culture, and the final value represents the mean of three independent experiments.

RESULTS

CbpD is highly expressed during competence. As described above, we have previously reported that an unidentified choline-binding protein must be important for competence-induced cell lysis (35, 36). Since CbpD is a competence-specific choline-binding protein that is dispensable for transformation, we considered it a likely candidate for this hitherto-unidentified component of the lysis mechanism. Previous studies using microarray analysis have shown that transcription of the *cbpD* gene increases upon competence induction and that *cbpD* belongs to the late competence genes (26, 32). To verify this, and to determine the basal level of transcription in noncompetent cells, we inserted a *lacZ* reporter gene downstream of the *cbpD* promoter. Our results show that transcription of the *cbpD* gene is very low or possibly absent in noncompetent cells, but is highly induced during competence (Fig. 1). The level of β-galactosidase expression is somewhat reduced at an OD_{550} of 0.5 compared an OD_{550} of 0.4. This is in accordance with the fact that an increasing fraction of the pneumococcal population becomes refractory to CSP at high cell density (13). Interestingly, we and others have found that the efficiency of transformation is not reduced in a *cbpD* mutant (26) (unpublished results). Thus, even though CbpD clearly is a competence-specific protein, it is not required for DNA binding, processing, uptake, or recombination.

Deletion of *cbpD* strongly reduces competence-induced cell lysis and DNA release. To directly test our hypothesis that CbpD is important for competence-induced cell lysis, we deleted the *cbpD* gene from the EK4166 strain and compared the resulting Δ*cbpD* mutant (EK4168) to the parental strain. Experimentally, cell lysis was detected as release of cytoplasmic β-galactosidase to the growth medium. The EK4166 strain is negative for endogenous β-galactosidase activity but harbors a chromosomally located transcriptional fusion between the *hirL* gene (GenBank accession no.AAL00764) and the *Escherichia coli lacZ* reporter gene (35). Since the *hirL* gene is highly expressed throughout the exponential growth phase, EK4166 and EK4168 cells have a high cytoplasmic content of β-galactosidase during this period. In addition, the two strains lack a functional CSP transporter (ComA) and can for this reason develop the competent state only if synthetic CSP is added to the culture.

To compare the properties of the Δ*cbpD* mutant (EK4168) and its parental strain (EK4166), we induced competence at cell densities ranging from an OD_{550} of 0.1 to 0.5 and measured the amount of β-galactosidase released to the growth medium after 30 min. The result of a representative experiment is presented in Fig. 2. It shows that almost no β-galactosidase activity was detected in sterile filtered culture supernatants from the Δ*cbpD* mutant, demonstrating that deletion of the *cbpD* gene has a dramatic effect on competence-induced cell lysis.

Competence-induced cell lysis is believed to increase the efficiency of horizontal gene exchange by providing extracellular DNA that can be taken up by competent recipient cells. We therefore considered it important to develop an assay that would allow us to directly measure DNA release from lysing cells in a competent culture of pneumococci. The obvious choice was to employ real-time PCR, a technique that makes it feasible to accurately compare the amount of extracellular DNA in competent and noncompetent cultures.

The primers and probes used for real-time PCR were directed against the pneumolysin gene of *S. pneumoniae*. Our results show that the content of extracellular DNA in a com-

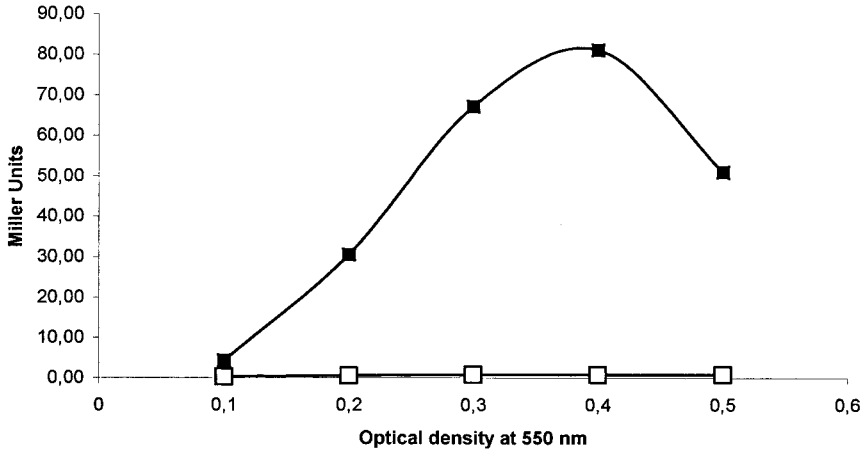

FIG. 2. Comparison of competence-induced release of cytoplasmic β-galactosidase from wild-type *S. pneumoniae* cells and a mutant lacking a functional *cbpD* gene. Bacterial cultures of strains EK4166 and EK4168 were collected at different cell densities (OD_{550} of 0.1 to 0.5) and induced to competence by addition of 250 ng/ml of synthetic CSP-1. After 30 min at 30°C, culture supernatants were harvested by centrifugation followed by sterile filtration. The supernatants were subsequently assayed for β-galactosidase activity. Solid squares, supernatants from EK4166 cells; open squares, supernatants from EK4168 cells. The experiment has been repeated several times with similar results. Genotypes of strains: EK4166, *comA egb hirL*::*pEVP3*; and EK4168, *comA egb hirL*::*pEVP3 cbpD*.

petent culture of strain CP1415 (*comA*) is more than a hundredfold higher than in a noncompetent culture of the same strain (Fig. 3). Samples were withdrawn from the cultures 30 min after addition of CSP in order to make the measurement within the period of competence. Transformation reaches a maximum 20 to 25 min after the addition of CSP and then gradually decays during the following 20 to 30 min (13). In a corresponding experiment, carried out with the Δ*cbpD* mutant strain termed L3, the results revealed only a fivefold increase in extracellular DNA content in competent cultures relative to their noncompetent counterparts (Fig. 3). This drastic reduction in CSP-induced release of DNA is in good agreement with the data presented in Fig. 2. Together the results obtained with real-time PCR and the β-galactosidase release assay clearly

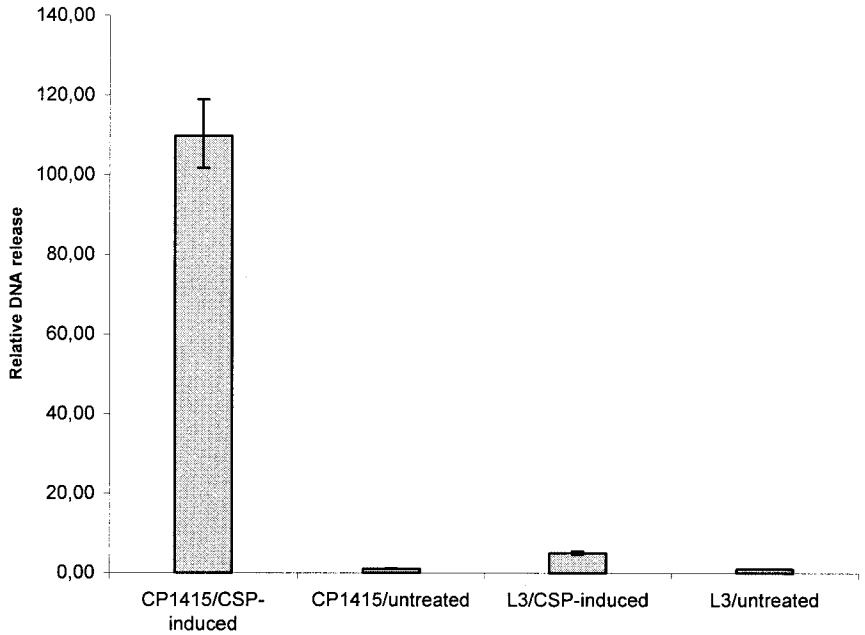

FIG. 3. Quantitative comparison of competence-induced DNA release in wild-type and Δ*cbpD* cells. When the bacterial cultures reached an OD_{550} of 0.3, 15 pg/ml control DNA (pLNO2HM1) and 0.5 μg/ml salmon sperm DNA were added. The cultures were immediately split in two, and one half of the culture was induced to competence by addition of 250 ng/ml of synthetic CSP while the other was kept untreated. After 30 min of incubation at 30°C, supernatants were collected by centrifugation and sterile filtered. Subsequently, the collected supernatants were used in a real-time PCR assay with primers and probes directed against the pneumolysin gene or the *hm1* gene. The amount of released DNA in untreated (noncompetent) cultures was set to 1. Data are plotted as the means of three independent experiments with error bars representing the standard deviations. Genotypes of strains: CP1415, *comA*; and L3, *comA cbpD*.

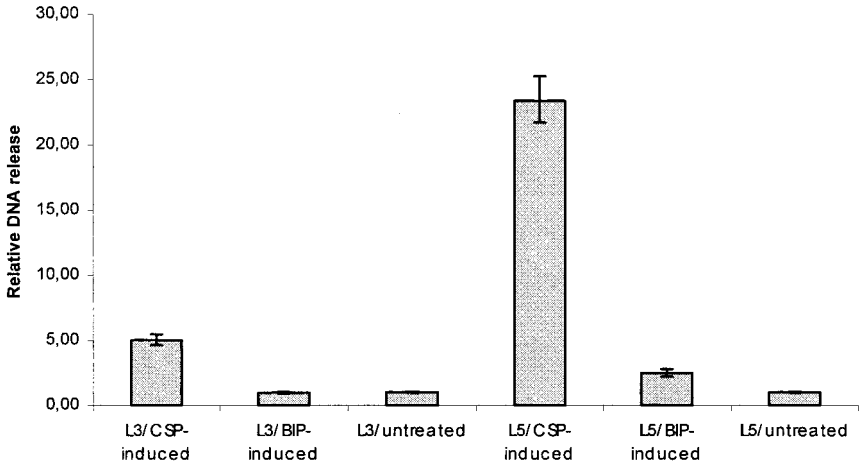

FIG. 4. Ectopic expression of CbpD. When cultures of the L3 and L5 mutants reached an OD_{550} of 0.3, 15 pg/ml control DNA (pLNO2HM1) and 0.5 μg/ml salmon sperm DNA were added. The cultures were immediately split in three and induced with 250 ng/ml CSP-1, 250 ng/ml BIP-1, or kept untreated. After 30 min incubation at 30°C, supernatants were collected by centrifugation followed by sterile filtration. Subsequently, the collected supernatants were used in a real-time PCR assay with primers and probes directed against the pneumolysin gene or the *hm1* gene. The amount of released DNA in untreated (noncompetent) cultures was set to 1. Data are plotted as the means of three independent experiments with error bars representing the standard deviations. Genotypes of strains: L3, *comA cbpD*; and L5, *comA cbpD qsrA*::*cbpD*.

demonstrate that CbpD is a key component of the lysis mechanism.

Ectopic expression of CbpD. As removal of CbpD almost abolished competence-induced cell lysis, we considered it of interest to determine if ectopic expression of CbpD in noncompetent cells would provoke cell lysis at a level comparable to that observed for competent CP1415 cells (see Fig. 3). In other words, is CbpD the only gene product expressed during competence that is required for cell lysis, or are additional competence specific gene products needed as well?

To answer this question, we inserted the *cbpD* gene into the chromosome behind the inducible promoter of the *qsrAB* operon. The *qsrAB* genes (accession no. AAL00365 and AAL00366) encode an ABC-transporter of unknown function. Recently, we showed that transcription from this promoter is induced by both CSP-1 and BIP-1 (15). CSP-1 is the competence-stimulating peptide produced by pneumococci possessing the *comC-1* allele, such as the *S. pneumoniae* strain Rx used in this study (28). The BIP-1 peptide pheromone shares no sequence homology with CSP-1 and is not able to induce competence in *S. pneumoniae* strain Rx. It is part of a homologous but separate quorum-sensing system (BlpABCSRH) that regulates production of bacteriocins in strain Rx (8, 30). The inducible direct repeat promoter upstream *qsrA* binds both BlpR and ComE and can therefore be activated by addition of CSP-1 or BIP-1 to the growth medium.

To obtain the same level of CbpD expression with both peptide pheromones, two point mutations were introduced in the right repeat as previously described for the OE4145 mutant constructed by Knutsen et al. (15). The resulting strain (L5) was then used to compare the amount of DNA release mediated by CbpD when this protein was expressed together with or separate from the other competence proteins. Our results clearly show that other proteins expressed during competence are needed in addition to CbpD to obtain DNA release comparable to that observed for the CP1415 strain (Fig. 4). Only a very small, but probably significant, increase in DNA release was detected when CbpD was expressed alone. In sum, the data presented in Fig. 4 show that even though CbpD is a crucial component of the lysis mechanism, at least one additional competence protein must be involved.

LytA and LytC. We have previously reported that significantly less β-galactosidase is released from a *lytA* mutant during competence development than from the wild-type positive control (36). The difference was two- to fourfold depending on the density of the cell culture when competence was induced. In the present work, we used real-time PCR to directly compare the amount of pneumococcal DNA present in the medium of competent and noncompetent cultures of a *lytA* mutant (H3). At an OD_{550} of 0.3, the culture was split in two, and 250 ng/ml of CSP was added to one of them. After 30 min incubation at 37°C, samples were withdrawn, briefly centrifuged, sterile filtered, and stored at −80°C for subsequent real-time PCR analysis. Our results show that there is about 50-fold more DNA present in the growth medium of the competent H3 culture than in the noncompetent H3 culture run in parallel (Fig. 5). Thus, competence-induced cell lysis is reduced about twofold in a *lytA* mutant compared to the wild-type CP1415 strain (Fig. 3). In contrast, a *lytC* mutant (H5) analyzed in the same way as H3 was not affected with respect to DNA release (Fig. 5). The difference in extracellular DNA concentration between competent and noncompetent cultures of the *lytC* mutant was found to be more than a hundredfold, i.e., the same as for the wild-type CP1415 strain (Fig. 3).

DISCUSSION

In the present work, we have obtained strong evidence that CbpD is a key component of the competence-induced cell-lysis mechanism. Computer-aided analysis of the 448-amino-acid sequence of CbpD shows that it is composed of three different domains plus a signal sequence at the N-terminal end. Down-

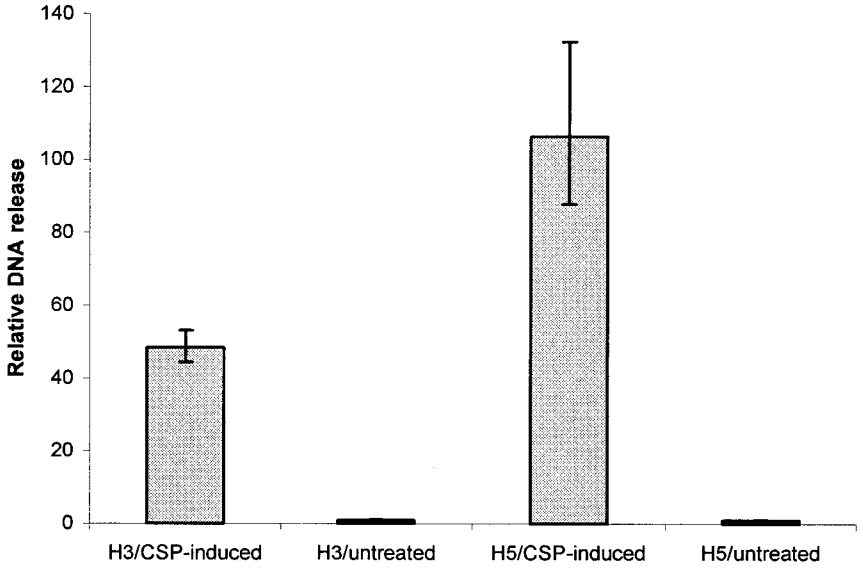

FIG. 5. Competence-induced DNA release in Δ*lytA* and Δ*lytC* mutants. Cultures of the H3 and H5 mutants were incubated at 37°C until they reached an OD_{550} of 0.3. Then 15 pg/ml control DNA (pLNO2HM1) and 0.5 μg/ml salmon sperm DNA were added. The cultures were immediately split in two, transferred to 30°C, and induced with 250 ng/ml CSP or kept untreated. After 30 min incubation at 30°C, supernatants were collected by centrifugation and sterile filtered. Subsequently, the collected supernatants were used in a real-time PCR assay with primers and probes directed against the pneumolysin gene or the *hm1* gene. The amount of released DNA in untreated (noncompetent) cultures was set to 1. Data are plotted as the means of three independent experiments with error bars representing the standard deviations. Genotypes of strains: H3, *comA lytA*::*pEVP3* Nov^r; and H5, *comA egb lytC*::*pEVP3*.

stream of the signal sequence is a CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) domain consisting of about 150 amino acids followed by two SH3 domains and a choline-binding domain with three or four choline-binding repeats (1, 3, 31). The function of choline-binding domains has been thoroughly studied. They are known to anchor proteins possessing such domains to the cell wall of *Streptococcus pneumoniae* by binding noncovalently to phosphorylcholine moieties linked to teichoic and lipoteichoic acid (9, 33). Consequently, CbpD must be considered an extracellular protein that is noncovalently attached to the pneumococcal cell wall. This is in agreement with the results of Gosink et al. (11), who used specific antibodies to detect CbpD in choline eluates from pneumococcal cells.

The CHAP family of enzymes consists predominantly of bacterial and phage amidases (1, 3, 31). Phylogenetic analysis has shown that CbpD belongs to the AcmB/LytN subfamily of these enzymes (1). Members of this subfamily are mostly found among gram-positive bacteria and function as cell wall hydrolases. Consequently, it is reasonable to assume that the CHAP domain of CbpD functions as a murein hydrolase as well. Work is in progress at our laboratory to verify this experimentally. The function of bacterial SH3 domains is not known. They are however often associated with CHAP domains and other cell wall-degrading enzymes. It is therefore tempting to speculate that SH3 domains are involved in attaching these enzymes to their substrate by binding directly either to murein or to other cell wall components. Indeed, it has been shown that the SH3 domain of lysostaphin targets this bacteriolytic enzyme to the cell wall of *Staphylococcus aureus* (2). Thus, available data on CbpD and its CHAP, SH3, and choline-binding domains indicate that CbpD is a cell wall-anchored murein hydrolase that functions as a competence-specific lysin.

Interestingly, our results strongly indicate that overexpression of CbpD alone is not sufficient to obtain effective cell lysis and that expression of additional competence-induced genes is required (Fig. 4). Transcriptome analyses have shown that CSP induces the expression of a large number of proteins apparently dispensable for DNA binding, uptake, and recombination. It is therefore likely that one or more of these proteins are part of the lysis mechanism. We have previously found that disruption of the gene encoding the autolysin LytA reduce competence-induced cell lysis two- to fourfold (36).

In the present paper, we used real-time PCR two quantify release of DNA in competence-induced and noncompetent cultures of pneumococci lacking a functional *lytA* gene. With this method, a twofold reduction of DNA release was observed in the *lytA* mutant. These results suggest that LytA acts together with CbpD to degrade the cell wall of cells undergoing lysis during competence. As only a fraction (5 to 20%) of the cells in a competent batch culture will lyse within the peak of competence, i.e., during the 30 min following addition of CSP (35), a mechanism must exist that protects the majority of the cells in the population or makes only a subfraction of the cells susceptible to lysis. This mechanism is not understood but may involve additional proteins required to control the activity of CbpD and LytA.

Other important questions regarding the mechanism of lysis remain unanswered as well. Initially,, we hypothesized that competence-induced release of DNA takes place by autolysis, in a process where some altruistic cells in the population commit suicide to provide DNA to the others. Recently, however,

we discovered that heterolysis (the lysis of one bacterium brought about by another) takes place in a competent *S. pneumoniae* culture and that heterolysis probably requires cell-to-cell contact (36). This finding does not necessarily prove that our original hypothesis is wrong. It is possible that heterolysis is a secondary effect of autolysis, caused by murein hydrolases released from cells undergoing autolysis. At present, it is therefore not clear whether autolysis or heterolysis is the principal cell lysis mechanism operating in a competent pneumococcal cell culture. Hopefully, future elucidation of the exact function of CbpD will help solve this question and lead to a better understanding of the biological significance of competence-induced cell lysis.

In a previous study, we used a β-galactosidase release assay to show that disruption of the *lytC* gene has no effect on competence-induced cell lysis (36). The *lytC* gene encodes a choline-binding autolytic lysozyme that does not belong to the CSP-responsive competence genes (10). In the present study, we employed real-time PCR to measure CSP-induced DNA release from the *lytC* mutant. Again we were not able to detect any difference between the *lytC* mutant (H5) and the wild-type strain CP1415 (Fig. 3 and Fig. 5). In contrast to our results, Moscoso and Claverys (23) found that inactivation of the *lytC* gene affects DNA release to the same extent as inactivation of *lytA*. They also found that CSP-induced DNA release is almost abolished in a *lytA lytC* double mutant. Our results, on the other hand, strongly indicate that CbpD is a key component of the lysis mechanism and that this putative murein hydrolase acts together with LytA to degrade the pneumococcal cell wall.

At present, there is no obvious explanation for these conflicting results. However, as pointed out by Moscoso and Claverys, it is possible that the observed discrepancy results from the use of different strains and media. Another possibility is that the conflicting results are due to different experimental designs. We have measured release of DNA or β-galactosidase in samples collected 30 min after addition of CSP, i.e., within the period of competence. In contrast, Moscoso and Claverys measured DNA release at multiple time points for at least 3 h post-competence induction, i.e., for several hours after the competent state had been shut off. They found that DNA release in cultures that had gone through a period of competence continued to increase throughout the exponential and into the stationary phase. No such release was seen in parallel cultures that had remained noncompetent during the experiment.

In our opinion, it is conceivable that different mechanisms are behind early and late DNA release. Early DNA release takes place during the period of competence and is, according to our results, strongly dependent on CbpD. Cells that lyse and disintegrate during this early period will shed much of their enzyme content into the environment, presumably including the autolysins LytA and LytC. Reminiscent of a chain reaction, it is possible that LytA and LytC released in this way will attack and lyse new cells, leading to release of more autolysins, and so forth. This scenario is not pure speculation, as we have observed that a wild-type strain, which always undergoes autolysis in late stationary phase due to the activity of LytA, will provoke lysis of a *lytA* mutant strain when the two strains are grown in a mixed culture (unpublished observation). This experiment clearly demonstrates that pneumococcal cells that lyse and fall apart release components (presumably autolysins) that attack and lyse other cells in the same culture. Consequently, it is possible that late DNA release is mainly caused by autolysins that are continuously released by disintegrating cells. This would explain why early DNA release requires CbpD and not LytC, whereas late DNA release depends mainly on the joint action of the two autolysins LytA and LytC.

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