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LytF, a Novel Competence-Regulated Murein Hydrolase in the Genus *Streptococcus*

Kari Helene Berg, Hilde Solheim Ohnstad, and Leiv Sigve Håvarstein

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

Streptococcus pneumoniae **and probably most other members of the genus** ***Streptococcus*** **are competent for natural genetic transformation. During the competent state,** ***S. pneumoniae*** **produces a murein hydrolase, CbpD, that kills and lyses noncompetent pneumococci and closely related species. Previous studies have shown that CbpD is essential for efficient transfer of genomic DNA from noncompetent to competent cells** ***in vitro*****. Consequently, it has been proposed that CbpD together with the cognate immunity protein ComM constitutes a DNA acquisition mechanism that enables competent pneumococci to capture homologous DNA from closely related streptococci sharing the same habitat. Although genes encoding CbpD homologs or CbpD-related proteins are present in many different streptococcal species, the genomes of a number of streptococci do not encode CbpD-type proteins. In the present study we show that the genomes of nearly all species lacking CbpD encode an unrelated competence-regulated murein hydrolase termed LytF. Using** ***Streptococcus gordonii*** **as a model system, we obtained evidence indicating that LytF is a functional analogue of CbpD. In sum, our results show that a murein hydrolase gene is part of the competence regulon of most or all streptococcal species, demonstrating that these muralytic enzymes constitute an essential part of the streptococcal natural transformation system.**

In streptococcal species belonging to the mitis phylogenetic group, such as *Streptococcus gordonii*, *Streptococcus pneumoniae*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus infantis*, *Streptococcus cristatus* and *Streptococcus sanguinis*, competence for natural genetic transformation is regulated by a signaling peptide of the competence-signaling peptide (CSP) type (17, 23). Streptococcal strains and species belonging to this group produce a number of distinct CSPs with different primary structures and specificities (24). The CSP precursor, ComC, is ribosomally synthesized and contains a double-glycine-type leader that is removed concomitant with export by a dedicated ABC transporter, termed ComA (14, 15, 22). In the case of *S. sanguinis*, the ComC leader peptide is not of the GG type, but the mature secreted peptide pheromone is clearly related to the CSPs produced by the other mitis group streptococci (17). The protein that translocates the CSP peptide across the cytoplasmic membrane of *S. sanguinis* has not been identified. The extracellular concentration of CSP is monitored by the ComDE two-component regulatory system (16, 37). At the critical external concentration of CSP, phosphorylated ComE activates expression of the alternative sigma factor ComX and other early competence genes. ComX then activates transcription of the late competence genes, some of which encode proteins involved in DNA processing, uptake, and recombination (32).

In *S. pneumoniae* only 23 of more than 100 CSP-inducible genes are required for transformation (6, 39). Two of the dispensable genes, *cbpD* and *comM*, are key players in a competence-associated mechanism called fratricide (13, 18, 26). When pneumococci develop the competent state, they synthesize and secrete the late gene product CbpD. This murein hydrolase functions as a weapon, termed fratricin, that lyses and kills noncompetent cells. In other words, the competent pneumococci act as predators that attack and lyse noncompetent target cells present in the same culture. The competent cells that secrete CbpD protect themselves by producing the immunity protein ComM, a membrane-embedded protein that neutralizes the muralytic activity of CbpD by an unknown mechanism (18). Since ComM is the product of an early competence gene, noncompetent pneumococci lack this immunity protein and are therefore susceptible to CbpD. Pneumococcal CbpD consists of one catalytic domain and two types of cell wall binding domains. The N-terminal CHAP (cysteine histidine-dependent aminohydrolase/peptidase) domain most likely functions either as an amidase that disrupts the *N*-acetylmuramyl-L-Ala bond or as an endopeptidase that cleaves within the peptide part of peptidoglycan (1, 30, 44). At the C-terminal end of CbpD, there are four choline-binding repeats. These repeats, which together constitute a choline-binding domain (CBD), anchor CbpD noncovalently to choline-decorated teichoic acid present in the pneumococcal cell wall (46). In the middle of the protein, between the CHAP and CBD domains, CbpD contains two Src homology 3 (SH3)-binding (SH3b) domains. These domains bind to the peptidoglycan part of the cell wall (8).

As fratricide is coregulated with natural transformation, we speculated that it evolved to facilitate acquisition of homologous donor DNA from other pneumococcal strains and related streptococcal species. Recently, we obtained strong evidence that this is indeed the case. In experiments carried out *in vitro*, we showed that the fratricide mechanism enhances gene exchange between pneumococci and also demonstrated that it has a large positive impact on the efficiency of gene transfer from the commensals *S. mitis* and *S. oralis* to *S. pneumoniae* (25). The fact that pneumococcal CbpD stimulates interspecies gene exchange *in vitro* shows that it is able to lyse *S. mitis* and *S. oralis*. This cross-species activity was expected as CbpD proteins from *S. mitis* and *S. oralis* are highly homologous to pneumococcal CbpD (Fig. 1). In addition,

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Address correspondence to Leiv Sigve Håvarstein, sigve.havarstein@umb.no.
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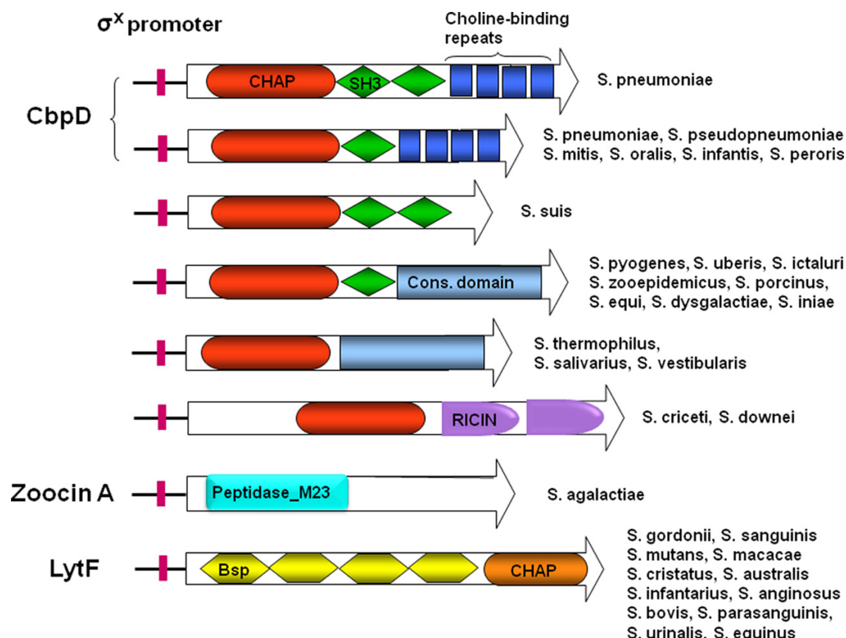

FIG 1 Domain organization of σ^X-controlled murein hydrolases from different species of streptococci: CHAP, cysteine, histidine-dependent amidohydrolases/peptidases; SH3, binds peptidoglycan (8), choline-binding repeats, bind choline residues linked to teichoic acid; conserved domain, uncharacterized domain that probably mediates binding to the cell wall of target cells; RICIN, carbohydrate binding domain; peptidase M_23, zinc metallopeptidase with a range of specificities. The gene from each streptococcal species that corresponds to the depicted gene product has the following locus tag: spr2006 (*S. pneumoniae* R6), SPPN_11215 (*S. pseudopneumoniae* IS7493), SM12261_0760 (*S. mitis* NCTC 12261), SOR_1962 (*S. oralis* Uo5), HMPREF9967_0542 (*S. infantis* SK 1076), HMPREF9180_0030 (*S. peroris* ATCC 700780), SSUBM407_1976 (*S. suis* BM 407), SPy_0031 (*S. pyogenes* M1 GAS), SUB0048 (*S. uberis* 0140J), Sict7_010100010455 (*S. ictaluri* 707-05), SeseC_00035 (*S. equi* subsp. *zooepidemicus* ATCC 35246), STRPO_1436 (*S. porcinus* strain Jelinkova 176), SEQ_0031 (*S. equi* subsp. *equi* 4047), SDD27957_00205 (*S. dysgalactiae* subsp. *dysgalactiae* ATCC 27957), no locus tag assigned (*S. iniae*), stu0039 (*S. thermophilus* LMG 18311), STRSA0001_1425 (*S. salivarius* SK126), HMPREF9425_1781 (*S. vestibularis* ATCC 49124), ScriH_010100000025 (*S. criceti* HS-6),HMPREF9176_1241 (*S. downei* F0415), SAG0031 (*S. agalactiae* 2603V/R), SGO_2094 (*S. gordonii* strain Challis), SSA_0036 (*S. sanguinis* SK 36), SMU.836 (*S. mutans* UA159), SmacN1_010100005870 (*S. macacae* NCTC 11558), HMPREF9422_1401 (*S. cristatus* ATCC 51100), HMPREF9421_0380 (*S. australis* ATCC 700641), STRINF_01688 (*S. infantarius* subsp. *infantarius* ATCC BAA-102), HMPREF9966_1370 (*S. anginosus* SK52), HMPREF9319_1268 (*S. bovis* ATCC 700338), HMPREF0833_11509 (*S. parasanguinis* ATCC 15912), Suri2_010100004140 (*S. urinalis* 2285-97), and HMPREF0819_0723 (*S. equinus* ATCC 9812).

all three species possess choline-decorated wall teichoic acid (WTA) and lipoteichoic acid (LTA), designated C-polysaccharide and F-polysaccharide, respectively (12). Recently, an *in silico* survey revealed that genes encoding CbpD-like proteins are present in the genomes of many species belonging to the genus *Streptococcus* (5). The CbpD-like proteins were identified on the basis of their highly conserved N-terminal CHAP domains. Interestingly, their C-terminal halves are not conserved, suggesting that the cell wall binding specificities of the various CbpD-like proteins differ. In all cases ComX-binding motifs (often referred to as com- or cin-boxes [3, 38]) were present in the promoter regions of the genes (5). Consequently, it is reasonable to assume that the CbpD-like proteins serve the same function as pneumococcal CbpD, namely, to kill and lyse noncompetent streptococci to provide homologous donor DNA for the competent attacker cells. Many of the species harboring these CbpD-like genes have never been demonstrated to be competent for natural transformation. However, recently it has been reported that natural transformation is probably much more widespread in the genus *Streptococcus* than previously recognized (19, 34).

Although many streptococcal species possess genes encoding CbpD-like proteins, homologues of *cbpD* genes are not found in naturally transformable streptococci such as *S. gordonii*, *S. sanguinis*, and *S. mutans*. Does this mean that these species lack the fratricide mechanism? To address this question we searched the genomes of these bacteria for possible CbpD substitutes. Here, we report the identification of a novel murein hydrolase, termed LytF, which is unrelated to CbpD but apparently serves the same function (Fig. 1). Intriguingly, most streptococci lacking a *cbpD* gene seem to have a *lytF* gene instead.

MATERIALS AND METHODS

Bacterial growth conditions and storage. Bacterial species, strains, and plasmids used in this work are described in Table 1. Bacterial cultures were grown at 37°C in Todd-Hewitt medium (Becton, Dickinson and Co.) (for *S. gordonii*, *S. thermophilus*, *S. mitis*, *S. oralis*, and *S. infantis*), C medium (28) (for *S. pneumoniae*), M17 supplemented with 0.5% glucose (GM17) (Oxoid) (for *Lactococcus lactis*), or brain heart infusion broth (Becton, Dickinson and Co.) supplemented with 5% horse serum (PAA Laboratories Inc.) (for *Streptococcus anginosus* [*Streptococcus milleri*], *S. cristatus*, *Streptococcus constellatus*, and *Streptococcus pyogenes* and for *Staphylococcus aureus*). *S. sanguinis* was grown in pH-regulated Todd-Hewitt medium (pH 6.8) to avoid autoinduction of competence. Precultures of the bacteria were grown to an optical density at 550 nm (OD_{550}) of 0.3 and maintained as glycerol stocks at −80°C.

Construction of *S. gordonii* mutants. The sequences of all primers used are given in Table 2. DNA was introduced into recipient strains by natural transformation. Bacterial cultures were grown to an OD_{550} of ~0.2 and induced to the competent state by addition of 250 ng ml^{-1} synthetic Challis strain CSP (NH_2-DVRSNKIRLWWENIFFNKK-COOH). Then, the transforming DNA was added, and the cultures were further incubated for 120 min at 37°C. Selection of transformants was

TABLE 1 Bacterial species, strains, and plasmids

Species, strain, or plasmid	Genotype or relevant feature(s)[a]	Reference or source[b]
S. gordonii strains		
NCTC 7865^T		NCTC
AH2	NCTC 7865, but *comA*::pEVP3 Cm^r Rif^r through spontaneous conversion	48
NCTC 3165		NCTC
NCTC 7868	Strain Challis	NCTC
SGH139	NCTC 7868, but *comA*::pEVP3 Cm^r	This work
SGH24	SGH139 with a deletion of *sgo_2094* by replacement with the *aacA-aphD* kanamycin resistance gene; Cm^r Kan^r	This work
SGH25	SGH139, but Sm^r through spontaneous conversion; Cm^r Sm^r	This work
SGH37	SGH139, but Rif^r through spontaneous conversion; Cm^r Rif^r	This work
SGH43	SGH25, with a deletion of *sgo_2094* by replacement with the *aacA-aphD* kanamycin resistance gene; Cm^r Sm^r Kan^r	This work
SGH141	SGH25 with a deletion of *celB* by replacement with the *aacA-aphD* kanamycin resistance gene; Cm^r Sm^r Kan^r	This work
SGH142	SGH25 with a deletion of *comD* by replacement with the *aacA-aphD* kanamycin resistance gene; Cm^r Sm^r Kan^r	This work
Other species or strains		
E. coli DH5α		Invitrogen
E. coli BL21		Invitrogen
E. coli ECH12	DH5α harboring pHOG2	This work
E. coli ECH13	BL21 harboring pHOG2	This work
E. coli ECH142	DH5α harboring pAH1	This work
S. sanguinis SK36		M. Kilian
S. sanguinis SSH605	SK36 with a deletion of *ssa_0036* by replacement with the *aacA-aphD* kanamycin resistance gene; Kan^r	This work
S. anginosus NCTC 10713^T		NCTC
S. anginosus NCTC 10708		NCTC
S. constellatus NCTC 11325^T		NCTC
S. cristatus NCTC 12479^T		NCTC
S. infantis DSM 12492^T		DSMZ
S. mitis NCTC 12261		NCTC
S. oralis SK155		M. Kilian
S. pneumoniae RH14		7
S. pyogenes NCTC 8198^T		NCTC
S. thermophilus LMG 18311		ATCC[d] BAA-250
Staphylococcus aureus RN 4220		S. J. Foster
Lactococcus lactis NZ9000		27
Plasmids		
pEVP3	Carries chloramphenicol resistance gene (*cat*)	4
pAH1	pEVP3 with *comA* fragment; Cm^r	This work
pFW13	Carries the *aacA-aphD* kanamycin resistance gene	40
pRSET A		Invitrogen
pRSET/EmGFP	Carries the *gfp* gene; Amp^r	Invitrogen
pHOG2	pRSET A containing *lytF-gfp* fusion	This work

[a] Amp, ampicillin; Cm, chloramphenicol; Kan, kanamycin; Rif, rifampin; Sm, streptomycin.
[b] NCTC, National Collection of Type Cultures; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; ATCC, American Type Culture Collection.

carried out on Todd-Hewitt agar containing the appropriate antibiotic at the following concentrations: kanamycin (Kan) at 350 μg ml^{-1} or spectinomycin (Spc) at 150 μg ml^{-1}. Resistance to streptomycin (Sm) or rifampin (Rif) was obtained through spontaneous conversion by selection on agar plates containing 1 mg ml^{-1} streptomycin or 20 μg ml^{-1} rifampin, respectively. Transformation of *S. sanguinis* was performed in the same way, by adding 250 ng ml^{-1} synthetic CSP13b (NH_2-DLRGVPNP WGWIFGR-COOH).

Strain SGH139 was derived from *Streptococcus gordonii* NCTC 7868 by insertion duplication mutagenesis targeting the *comA* gene (*sgo_2097*). An ~1,300-bp region of *comA* was amplified from NCTC 7868 using the primers a1 and a2, containing BamHI and KpnI sites at their 5′ ends, respectively. The fragment was digested with BamHI and KpnI and ligated into the pEVP3 vector, precleaved with the same enzymes. The resulting plasmid (pAH1) was transformed into electrocompetent *Escherichia coli* DH5α cells, according to standard procedures (45), resulting in ECH142. pAH1 was purified from ECH142 and transformed into *S. gordonii* NCTC 7868 by natural transformation, resulting in the insertion duplication mutant SHG139 that contains a disrupted *comA* gene.

Strains SGH25 and SGH37 were derived from SGH139. Spontaneous resistance to streptomycin (SGH25) or rifampin (SGH37) was obtained by selection on agar plates containing 1 mg/ml streptomycin or 20 μg/ml rifampin, respectively.

Strain SGH43 was derived from SGH25 by replacing *lytF* (*sgo_2094*)

TABLE 2 Primers[a]

Primer	Oligonucleotide sequence (5′ → 3′)
a1	ATTAGGATCCAT8AT8GAYAC8TAYGT8CC8GA
a2	TTAAGGTACC8CC8GA8AT8CC8GC8CCRTC
hso36	AGCAATGGCTAGCAATGAACAGTTTAGCCCATCCCAAC
hso38	CTAATTTTCAAGTAACACGCCCAACGGTAGTGAGCAAGGGCGAGGAG
hso39	AGCAATCTCGAGTTACTTGTACAGCTCGTCCATGC
hso50	CTTGATAATCGTCTCAACTGACT
hso51	AGCTTAAGATCTAGAGCTCGAGGATCAAGGTTGCTGAAAGCAGTAAGAA
hso52	GGAAATATTCATTCTAATTGGTAAGCGCCCCTATCATTTAGCACCTCCCT
hso53	AATGCTACGATTGCTTCACAAAAT
kana-f	ATCCTCGAGCTCTAGATCTTAAGCTT
kana-r	ACTCCGGATGCATATGCATGCT
khb113	CTACAGGGAAGAGACCAAGG
khb114	CAAGCTTAAGATCTAGAGCTCCTTGGATAATGCCAGATTAGG
khb115	GAGCTCTAGATCTTAAGCTTG
khb116	CTCCGGATGCATATGCATGC
khb117	GCATGCATATGCATCCGGAGGCTGTGTGGTATAATATAGAGAAC
khb118	GTCAAGTCAAAAATATTGTCCTG
khb121	GTGGTAGTTTAGGTTTGTCTG
khb122	CAAGCTTAAGATCTAGAGCTCTGATATTACCATTATTAAACCCC
khb123	GAAAAGAAATTTATAAGAGGACTTAAGTAGGCTTCTCAACAAAAGGAG
khb124	CTGCTATTGTCCCATAAAATTC
ss1	CACCTGTATTGGGTCCCTTG
ss2	AGCTTAAGATCTAGAGCTCGAGGATCTGGGCCCACCTGTATTATTC
ss3	GGAAATATTCATTCTAATTGGTAAGCGCAAGTCGCTTTCGCACTTCTC
ss4	TTTCTATCAGACGGCCTTGC

[a] All primers were designed in the course of this study. 8, inosine.

with a kanamycin resistance gene (*aacA-aphD*). The kanamycin resistance gene was amplified from pFW13 (40) using the primers kanaF and kanaR. Fragments corresponding to the ~1,000-bp upstream and downstream regions of *sgo_2094* were amplified using the primer pairs hso50/hso51 and hso53/hso54, respectively. The upstream and downstream fragments were then fused to the 5′ and 3′ ends of the kanamycin cassette by overlap extension PCR (21), using the primers hso50 and hso54. The resulting PCR product was transformed into the SGH25 strain by natural transformation, giving rise to the SGH43 strain.

Strain SGH141 was constructed by replacing the *celB* gene (*sgo_1601*) in SGH25 with the kanamycin resistance gene referred to above. The primer pair khb115/khb116 was used to amplify the kanamycin resistance gene from pFW13. Fragments corresponding to the ~1,000-bp upstream and downstream regions of *celB* were amplified using the primer pairs khb113/khb114 and khb117/khb118, respectively. These two fragments were subsequently fused to the 5′ and 3′ ends of the kanamycin resistance gene using the primers khb113 and khb118. The resulting PCR product was transformed into the SGH25 strain by natural transformation, giving rise to the SGH141 strain.

Strain SGH142 was derived from SGH25 by replacing the *comD* gene (*sgo_2146*) with the pFW13 kanamycin resistance gene, as described above. Fragments corresponding to the ~1,000-bp upstream and downstream regions of *comD* were amplified using the primer pairs khb121/khb122 and khb123/khb124, respectively. These two fragments were subsequently fused to the kanamycin resistance gene using the primers khb121 and khb124. The resulting PCR product was transformed into the SGH25 strain by natural transformation, giving rise to the SGH142 strain.

The pHOG2 plasmid was derived from the pRSETA expression vector (Invitrogen). The *S. gordonii sgo_2094* gene, except for the region encoding the CHAP domain, was amplified using the primers hso36 and hso37. Similarly, the emerald green fluorescent protein (EmGFP) gene was amplified from pRSET/EmGFP (Invitrogen) with the primer pair hso38/hso39. The two fragments were subsequently fused by overlap extension PCR using the primers hso36 and hso39. The resulting PCR construct was cleaved with the restriction enzymes XhoI and NheI and ligated into the pRSETA vector precleaved with the same enzymes. The resulting plasmid, pHOG2, was transformed into competent *E. coli* DH5α cells, giving rise to ECH12. Finally, pHOG2 was purified from the ECH12 strain and transformed into *E. coli* BL21, resulting in ECH13.

S. sanguinis strain SSH605 was constructed by replacing the *ssa_0036* gene in *S. sanguinis* SK36 with the kanamycin resistance gene described above. Fragments corresponding to the ~1,000-bp upstream and downstream regions of *ssa_0036* were amplified using the primer pairs ss1/ss2 and ss3/ss4, respectively. The upstream and downstream fragments were then fused to the 5′ and 3′ ends of the kanamycin cassette, using the primers ss1 and ss4. The resulting PCR product was transformed into *S. sanguinis* SK36 by natural transformation, giving rise to the *S. sanguinis* SSH605 strain.

Zymogram analysis. Zymogram analysis was used to detect muralytic activity in whole-cell extracts from competent and noncompetent cultures of *S. gordonii* and *S. sanguinis*. To make extracts from competent cells, bacterial cultures were first grown at 37°C until they reached an OD_{550} of ~0.2. Then, 250 ng ml^{-1} of the appropriate CSP was added. After further incubation at 37°C for 15 min, cultures were harvested, and cells from 10-ml samples were pelleted by centrifugation. Prior to electrophoresis, pellets were dissolved in 50 μl of SDS sample buffer and 50 μl of MilliQ-water and heated to 95°C for 10 min. Extracts from noncompetent cells were made in the same way, except that no CSP was added.

The zymogram assay was performed according to the method described by Leclerc and Asselin (31). Whole-cell extracts were separated by SDS-PAGE using a 4% stacking gel and a 10% resolving gel as described by Laemmli (29). Heat-killed substrate cells were mixed into the resolving gel solution before it polymerized. To prepare the substrate cells, 300-ml cultures were grown at 37°C to an OD_{550} of 0.2. Then, the cells were immediately harvested by centrifugation (10,000 × *g*) at 4°C for 10 min. The resulting pellet was washed once in 5 ml of ice-cold Tris-HCl (20 mM, pH 7.4) buffer supplemented with 100 mM NaCl. After cells were washed, they were suspended in 1.25 ml of Tris-HCl (1.5 M, pH 8.8) and heat treated for 10 min at 95°C.

Following electrophoresis at 2.5V cm^{-1}, the gels were washed in distilled H_2O (dH_2O) two times for 30 min each before the refolding buffer (50 mM NaCl, 20 mM $MgCl_2$, 0.5% Triton X-100, and 20 mM Tris-HCl, pH 7.4) was added. The gels were incubated in refolding buffer until lytic activity was observed as bands of clear zones in the opaque gel.

Cocultivation and transformation assays. Attacker (SGH25 and SGH43, Sm^r) and target (AH2, Rif^r) strains were grown at 37°C to an OD_{550} of ~0.2. SGH25 or SGH43 cells (volume, 0.5 ml) were then mixed with an equal volume of AH2 cells and 250 ng ml^{-1} of Challis CSP. Cocultivation of attacker and target cells was carried out at 37°C for 2 h, after which the samples were put on ice. Next, samples were serially diluted in 10-fold steps and spread on Todd-Hewitt agar plates supplemented with streptomycin (100 μg ml^{-1}) or streptomycin (100 μg ml^{-1}) and rifampin (2 μg ml^{-1}). The agar plates were incubated at 37°C for 15 to 17 h before the number of Sm^r and Sm^r Rif^r colonies was determined. Uninduced samples were run in parallel as negative controls. Cocultivation experiments involving the attacker strain SGH37 and the target strains SGH140 or SGH141 were performed as described above.

Purified genomic DNA was used to determine transformation efficiency of strains SHG25 and SGH43. Bacterial cultures of these strains were grown at 37°C until they reached an OD_{550} of 0.2. Next, 250 ng of Challis CSP was added to 1-ml samples of cells together with saturating amounts of genomic DNA (0.5 μg ml^{-1}) isolated from the rifampin-resistant AH2 strain. The cultures were further incubated at 37°C for 2 h and immediately put on ice. Then, the samples were serially diluted and spread on Todd-Hewitt agar plates containing streptomycin (100 μg ml^{-1}) or streptomycin (100 μg ml^{-1}) and rifampin (2 μg ml^{-1}). Unin-

duced samples were run in parallel as negative controls. Colonies were counted after anaerobic incubation of the plates at 37°C for 15 to 17 h.

Preparation of cell wall sacculi. Cell wall sacculi from *S. gordonii* strain Challis were prepared essentially as described by Reusch (43). In brief, cells were harvested from a Challis culture at an OD_{550} of 0.37, pelleted by centrifugation, and suspended in 3.3 ml of freshly prepared extraction fluid consisting of 1% SDS (wt/vol)–0.5% β-mercaptoethanol (vol/vol) (SDS-ME). Next, the cells were incubated at 85°C for 15 min, followed by dilution in dH_2O to a final volume of 5 ml. The cells were dispersed with an Ultra-Turrax T25 basic instrument (Ika-Werke) for 15 s at maximum speed and harvested by centrifugation at 17,500 × *g* at 4°C for 30 min. The resulting pellet was dissolved in 3.3 ml of hot (85°C) SDS-ME solution. Extraction of the pellet with hot SDS-ME solution was repeated for a total of five times. The extracted material was washed five times with dH_2O, once with 2.0 M NaCl, once with dH_2O, once with 2.0 M NaCl, and four times with dH_2O. The washed sacculi were then dissolved in 200 μl of phosphate-buffered saline (PBS), pH 7.4, and treated with trypsin (1.5 μg ml^{-1}) overnight at 37°C. Sacculi were harvested at 16,000 × *g* at 4°C, dissolved in 300 μl of 4% SDS, and incubated at 85°C for 15 min. After centrifugation of the sacculi suspension for 30 min at 16,000 × *g* at 4°C, the sacculi were resuspended, washed three times in dH_2O, and suspended in 300 μl of PBS. To remove covalently attached wall teichoic acid (WTA), sacculi were treated with 48% hydrofluoric acid for 48 h at 4°C, as described by Eldholm et al. (8). Purified sacculi were stored at −20°C.

Expression and purification of LytF-GFP. Construction of the pHOG2 expression vector, which contains a gene encoding the His-tagged LytF-GFP fusion protein under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 promoter, is described above. An overnight culture of the ECH13 strain carrying the pHOG2 vector was diluted to an OD_{600} of ~0.1 in 100 ml of Luria-Bertani broth (1% Bacto tryptone [wt/vol], 1% NaCl [wt/vol], and 0.5% Bacto yeast extract [wt/vol]). The ECH13 culture was incubated at 37°C until it reached an OD_{600} of 0.5. The culture was then induced with 1 mM IPTG and incubated for 2 h with shaking (200 rpm) at 30°C. Next, the cells were pelleted by centrifugation at 4°C, resuspended in 8 ml of PBS and lysed by repeated freezing and thawing in liquid nitrogen and a 42°C water bath. After cell debris was removed by centrifugation (70,000 × *g* for 10 min at 4°C), the supernatant was filtered through a 0.45-μm-pore-size filter. His-tagged LytF-GFP was purified from the resulting supernatant by the use of Protino Ni-TED columns (Macherey Nagel) as described by the manufacturer.

Epifluorescence microscopy. The bacterial culture used to study LytF-GFP binding was harvested at an OD_{550} of ~0.4. The pellet from 1 ml of culture was washed once in 1 ml of PBS and, after centrifugation, dissolved in 200 μl of PBS. Five microliters of this suspension was transferred to a glass slide and mixed with purified fusion protein dissolved in PBS containing 0.05% Tween 20. The suspension was spread thinly over the slide and incubated at room temperature for 8 min. Then, the slide was washed three times with PBS containing 0.05% Tween 20. Finally, after a coverslip was positioned over the sample, the slide was imaged using a Zeiss LSM 700 confocal microscope. The same protocol was used to study binding of purified LytF-GFP to sacculi.

RESULTS

Identification of LytF, a novel competence-induced murein hydrolase. The absence of genes encoding CbpD-like fratricins from the genomes of *S. gordonii*, *S. sanguinis*, *S. mutans*, and several other streptococcal species made us wonder whether these species produce alternative murein hydrolases that carry out the same or a similar function as pneumococcal CbpD. By performing BLASTP searches against the *S. gordonii* genome with pneumococcal CbpD, we identified a few proteins carrying CHAP domains that were distantly related to the CHAP domain used as a query sequence. Interestingly, the gene encoding one of these proteins, *sgo_2094*, had a com-box in its promoter region, indicating that it is part of the ComX regulon. By screening the literature we found that *sgo_2094* had been identified as a late competence gene by Vickerman et al. (50), who had used microarray analysis to study transcriptional changes in *S. gordonii* in response to CSP. *sgo_2094* encodes a ~58-kDa protein consisting of an N-terminal signal peptide, three Bsp-like (group B streptococcal secreted protein) domains (42), and a C-terminal CHAP domain (1, 44) (Fig. 1). Proteins with Bsp domains appear to occur only in members of the genus *Streptococcus*. In all cases described so far, Bsp domains are part of modular proteins that in addition carry a CHAP, amidase, or *N*-acetylmuramidase domain (30). It follows from these observations that Bsp modules might function as cell wall binding domains that attach the accompanying muralytic domain to its substrate. To determine whether the *sgo_2094* gene product functions as a competence-specific murein hydrolase, we performed zymogram analyses with total protein extracts prepared from CSP-induced and noninduced cells of *S. gordonii* strain Challis. To avoid spontaneous competence induction in cultures used for this experiment, Δ*comA* mutants (SGH139 and SGH24) were employed (Table 1). Protein extracts made from SGH139 and SGH24 cells were run on SDS-PAGE gels containing heat-killed cells of the SGH139 strain. The resulting zymogram revealed two clearing zones, corresponding to proteins with molecular masses of ~60 kDa and ~30 kDa, which were present only in the protein extract from CSP-induced cells (Fig. 2). Total protein extracts from an *S. gordonii* strain (SGH24) lacking both the *comA* and the *sgo_2094* genes were analyzed in parallel. In this case, the 60-kDa as well as the 30-kDa clearing zone was missing (Fig. 2). These results show that the *sgo_2094* gene product, LytF, encodes a murein hydrolase, and that the 30-kDa band probably represents a degradation product of the full-length LytF protein.

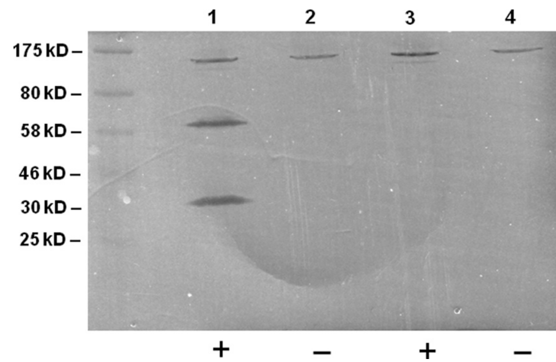

FIG 2 Murein-hydrolyzing activity in total extracts of *S. gordonii* strain Challis cells revealed by zymogram analysis. The dark bands represent clearing zones that result from degradation of Challis cells that have been incorporated in the SDS-PAGE resolving gel. Lane 1, extract from competence-induced SGH139 (Δ*comA*) cells; lane 2, extract from noncompetent SGH139 (Δ*comA*) cells; lane 3, extract from competence-induced SGH24 (Δ*comA* Δ*lytF*) cells; lane 4, extract from noncompetent SGH24 (Δ*comA* Δ*lytF*) cells.

The *ssa_0036* gene of *S. sanguinis* SK36 encodes a protein that is highly similar to Sgo_2094, except that the Ssa_0036 protein contains five instead of three predicted Bsp domains. The properties of the *S. sanguinis* version of the LytF protein were investigated by zymogram analysis essentially as described for the *S. gordonii* homologue. A clearing zone corresponding to a protein of ~70 kDa was detected in the protein extract prepared from CSP-induced *S.*

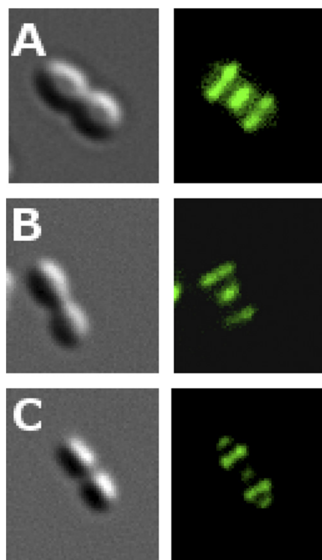

FIG 3 Binding of a LytF-GFP fusion protein to the surface of *S. gordonii* strain Challis cells. (A) Intact cells. (B) Sacculi. (C) Sacculi lacking teichoic acid. The fusion protein was constructed by exchanging the CHAP domain of LytF with GFP.

sanguinis SK36 cells. This zone was absent from extracts prepared from noninduced SK36 cells and a CSP-induced *S. sanguinis* mutant (SSH605) lacking the *ssa_0036* gene (results not shown). The predicted molecular mass of Ssa_0036 is ~70 kDa, which is in full agreement with the experimental data.

LytF localizes to the equator and septal regions of the *S. gordonii* cell wall. To determine whether the Bsp domains target LytF to the cell wall, we made a construct consisting of the Bsp portion of LytF fused to green fluorescence protein (GFP). A His tag was added to the N terminus of the recombinant protein to facilitate purification after overexpression in *Escherichia coli.* Fluorescence microscopy revealed that the Bsp-GFP fusion protein binds strongly to the *S. gordonii* cell wall and that it specifically localizes to the equatorial and septal regions (Fig. 3A). In a subfraction of the cells, binding to the polar regions was also observed. As the binding specificity of Bsp-type domains has not been studied before, it was of interest to identify the component of the cell wall that interacts with these domains. To investigate these matters, we prepared cell wall sacculi that consisted only of peptidoglycan and wall teichoic acid. A portion of the sacculus preparation was further treated with hydrofluoric acid to remove the teichoic acid component. Hence, the resulting sacculi consisted of pure peptidoglycan. Interestingly, the Bsp-GFP fusion protein bound to both types of sacculi, displaying a binding pattern identical to that observed with whole *S. gordonii* cells (Fig. 3B and C). These results demonstrate that Bsp domains recognize the peptidoglycan moiety of the streptococcal cell wall. Furthermore, since the Bsp-GFP fusion protein does not bind to the older section of the cell wall, Bsp domains must recognize structures that are present only in relatively newly synthesized peptidoglycan.

What is the biological role of LytF? LytF is encoded by a late competence gene and is expressed only during competence for natural transformation. What role does LytF play in this process? Although LytF and pneumococcal CbpD are only distantly related through their CHAP domains, they have several properties in common. They are both competence-induced murein hydrolases that bind to the equatorial/septal region of their respective species. As mentioned above, previous studies support the hypothesis that the biological function of CbpD is to lyse susceptible cells in order to release DNA that can be taken up by competent attacker cells (25). Considering the similar properties of CbpD and LytF, we speculated that they are functional analogues that serve the same or similar biological roles in their respective species. To test this experimentally, we performed cocultivation experiments between different strains of *S. gordonii* to compare the efficiency of gene transfer in the presence and absence of LytF. The mutants SGH25 (Δ*comA* Str^r) and SGH43 (Δ*comA* Δ*lytF* Str^r), derivatives of *S. gordonii* strain Challis, were used as the competence-induced attacker strains. As target strain we used the mutant AH2 (Δ*comA* Rif^r), a derivative of *S. gordonii* strain NCTC 7865. The Challis and NCTC 7865 strains produce CSPs with different primary structures. Challis-CSP (NH_2-DVRSNKIRLWWENIFFNKK-COOH) did not cross-induce competence in the NCTC 7865 strain, whereas a weak cross-inducing activity was observed when the Challis strain was subjected to 200 ng/ml of the NCTC 7865 CSP (NH_2-DIRHRINNSIWRDIFLKRK-COOH). Consequently, when Challis CSP is added to a mixed culture of Challis and NCTC 7865 cells, only the Challis cells will become competent for natural transformation. Cocultivation experiments showed that a Rif^r marker was transferred much more efficiently from NCTC 7865 to Challis cells when LytF was expressed by the competent attacker cells. The number of Str^r Rif^r doubly resistant transformants obtained with mixed cultures of SGH25 and AH2 cells was, on average, 100-fold higher than with corresponding cultures of SGH43 and AH2 cells (Table 3). This strongly indicates that LytF causes lysis of AH2 target cells, followed by release of DNA that can be taken up by competent SGH25 cells. To rule out the possibility

TABLE 3 Impact of LytF on the efficiency of gene transfer from donor to recipient cells during co-cultivation

DNA recipient strain	DNA source	Transformation efficiency during cocultivation (%)[d]	Transformation efficiency with genomic DNA (%)[d]
SGH25[a]	AH2	0.1 ± 0.019	
SGH43[a]	AH2	0.001 ± 0.0002	
SGH25[b]	AH2 DNA		0.87 ± 0.32
SGH43[b]	AH2 DNA		0.66 ± 0.16
SGH37[c]	SGH25	0.16 ± 0.046	
SGH37[c]	SGH141	0.08 ± 0.016	
SGH37[c]	SGH142	0.006 ± 0.001	

[a] The *S. gordonii* Challis strains SGH25 (Δ*comA* Str^r) and SGH43 (Δ*comA* Δ*lytF* Str^r) were induced to competence with Challis CSP and cocultivated with the noncompetent strain AH2 (Δ*comA* Rif^r). AH2, which is derived from *S. gordonii* strain NCTC 7865 does not respond to Challis CSP.

[b] Transformation of the SGH23 and SGH43 strains with purified genomic DNA from the AH2 strain. Saturating amounts of DNA were used.

[c] To determine if competent target cells are immune against LytF, the Challis attacker strain SGH37 (Δ*comA* Rif^r) was cocultivated with the Challis target strains SGH25 (Δ*comA* Str^r), SGH141 (Δ*comA* Δ*celB* Str^r), and SGH142 (Δ*comA* Δ*comD* Str^r) in the presence of CSP. As it lacks the DNA permease CelB, the SGH141 target strain is not able to take up DNA during competence. The SGH142 target strain contains a disrupted *comD* gene and will therefore remain noncompetent even in the presence of CSP.

[d] Transformation efficiency was estimated by dividing the number (CFU) of doubly resistant transformants by the total number (CFU) of competent recipient cells and multiplying by 100. Means and standard errors were calculated from three independent experiments.

TABLE 4 Specificity of LytF from *S. gordonii* strain Challis and *S. sanguinis* SK36 determined by zymogram analysis

Source of cells incorporated in SDS-PAGE gel as a substrate	Activity of LytF from:[a]	
	S. gordonii Challis	*S. sanguinis* SK36
S. gordonii NCTC 7865[T]	+	ND
S. gordonii NCTC 3165	+	+
S. anginosus NCTC 10713[T]	+	−
S. anginosus NCTC 10708	−	+
S. constellatus NCTC 11325[T]	−	+
S. sanguinis SK36	−	+
S. cristatus NCTC 12479[T]	−	+
S. pneumoniae RH 14	−	ND
S. mitis NCTC 12261	−	ND
S. oralis SK155	−	−
S. infantis DSM 12492[T]	−	−
L. lactis NZ9000	−	ND
S. aureus RN 4220	−	ND
S. thermophilus LMG 18311	+	ND
S. pyogenes NCTC 8198[T]	+	ND

[a] ND, not determined.

that this result was due to decreased transformability of the strain lacking LytF, the SGH25 and SGH43 strains were transformed with genomic DNA purified from the AH2 strain. The obtained transformation efficiencies show that there are no significant differences between the two strains (Table 3).

LytF fratricins have a limited target range. To gain insight into the specificity of LytF-type murein hydrolases, we performed a number of zymogram analyses in which cells from different species were used as substrates. The results showed that LytF from *S. gordonii* strain Challis was active against all *S. gordonii* strains tested but otherwise had a narrow muralytic spectrum (Table 4). The different strains and species used as substrates in the zymograms were also tested for their ability to bind the Bsp-GFP fusion protein described above. The fusion protein bound to all LytF-sensitive strains/species according to the pattern shown in Fig. 3A, while all LytF-resistant species tested negative in the Bsp-GFP binding assay (results not shown).

The muralytic spectrum displayed by LytF from *S. sanguinis* SK36 was different from, and included more species than, that of Challis LytF (Table 4). As the amino acid sequences of the CHAP domains of the two LytF proteins are 80% identical, the difference in target ranges is probably due to their N-terminal cell wall binding regions. The N-terminal part of Challis LytF contains three predicted Bsp domains, whereas the corresponding part of *S. sanguinis* LytF contains five such domains. In addition, large parts of the N-terminal regions of these proteins display relatively low homology. Together, our results show that similar to CbpD-type fratricins, the LytF type has a limited target range and is primarily directed against related bacteria.

Are competent *S. gordonii* cells immune to LytF? Competent *S. pneumoniae* bacteria produce the integral membrane protein, ComM, which protects them against the action of CbpD (18). ComM is encoded by an early competence gene and is consequently expressed about 5 min before CbpD. To address whether competent *S. gordonii* cells are immune to LytF, we carried out cocultivation experiments with competence-inducible and noninducible mutants of strain Challis. Three different experimental setups were used: experiment 1 using SGH37 (Δ*comA* Rif[r]) and SGH25 (Δ*comA* Str[r]), experiment 2 using SGH37 and SGH141 (Δ*comA* Δ*celB* Str[r]), and experiment 3 using SGH37 and SGH142 (Δ*comA* Δ*comD* Str[r]). The SGH141 strain lacks the DNA permease CelB (also called ComEC) and is therefore not able to take up DNA during competence, whereas SGH142 contains a disrupted *comD* gene and will remain noncompetent when subjected to CSP. In experiment 1, transfer of antibiotic resistance markers goes in both directions as both mutant strains become competent when CSP is added. In experiment 2, both mutant strains turn on expression of competence genes upon addition of CSP; however, strain SGH141 is not able to take up donor DNA due to its defective *celB* gene. As expected, the number of Rif Sm doubly resistant transformants was reduced 2-fold in experiment 2 compared to experiment 1 (Table 3). If competent *S. gordonii* cells produce an immunity protein that protects them against the muralytic activity of LytF, experiment 3 should have given rise to a higher number of doubly resistant transformants than experiment 2. Our results show that this was clearly not the case (Table 3). On the contrary, the transformation efficiency was found to be 13-fold higher in experiment 2 than in experiment 3. This result strongly indicates that *S. gordonii* cells lack a competence-induced immunity mechanism that protects them against LytF. The reduced transformation efficiency observed in experiment 3 is presumably due to decreased extracellular levels of LytF, resulting from the fact that LytF is produced by the SGH37 strain only in this cocultivation experiment.

DISCUSSION

Two major types of competence-associated murein hydrolases are present in the genus *Streptococcus* (Fig. 1). Those belonging to the CbpD-type have closely related N-terminal CHAP domains, while their C-terminal cell wall-targeting regions vary between species. Based on the structure of their C-terminal regions, six CbpD subtypes were identified (the six uppermost variants of CbpD depicted in Fig. 1). One of these, represented by the fratricins produced by *Streptococcus criceti* and *Streptococcus downei*, have not been reported before. Alignment of the C-terminal cell wall-targeting regions of fratricins belonging to the same CbpD subtype revealed that their sequences often have diverged considerably at the amino acid level, suggesting that fratricins belonging to the same subtype might possess different binding specificities and target ranges.

The C-terminal CHAP domains of LytF fratricins are highly conserved within the LytF group but are only distantly related to CbpD-type CHAP domains. Interestingly, the CHAP domains carried by LytF-type fratricins are highly similar to the CHAP domains of streptococcal PcsB proteins. Deletion of the *pcsB* gene or depletion of its gene product generates cells with multiple-division septa, demonstrating that PcsB is required for normal septum formation. It has been proposed that PcsB functions as a division hydrolase, but experimental proof of its muralytic activity is lacking (36, 41). The fact that LytF has murein hydrolase activity suggests that this is also the case for the PcsB proteins. The N-terminal regions of LytF and PcsB are totally unrelated, showing that these proteins serve different biological functions. All LytF-type fratricins have N-terminal regions consisting of two to five successive Bsp domains. The cell wall binding specificities of these regions, which have been shown to vary (Table 4), must be determined by the Bsp domains. Presumably, both the number

and structure of Bsp domains play a role in determining their binding properties and target ranges. Until now, proteins with Bsp domains have been found only in streptococci, where they are associated with murein hydrolases that carry enzymatic domains belonging to the PF05257 (CHAP), PF01520 (amidase_3), and PF01183 (Glyco_hydro_25) protein families (29).

BLASTP searches for homologues of CbpD or LytF in the genome of *Streptococcus agalactiae* were unsuccessful, suggesting that this species produces a different fratricin or lacks a competence-regulated cell lysis mechanism. Similar to many of the streptococcal species listed in Table 1, *S. agalactiae* has never been demonstrated to be competent for natural transformation. However, since all streptococci seem to possess the core competence genes and regulatory circuits that control their expression, it is reasonable to assume that most or all members of the genus are naturally transformable (19, 34). To investigate the possibility that *S. agalactiae* produces a competence-induced murein hydrolase that is unrelated to CbpD and LytF, we scanned its genome for alternative candidates. Intriguingly, we found that a gene encoding a close homologue of zoocin A, a protein previously identified as a muralytic bacteriocin, contains a cin-box in its promoter region. The presence of a ComX binding site upstream of the *S. agalactiae* version of zoocin A strongly indicates that it functions as a fratricin. The zoocin A bacteriocin, which is produced by some strains of *Streptococcus equi* subsp. *zooepidemicus*, is a D-alanyl-L-alanine endopeptidase that hydrolyzes the peptidoglycan cross bridges of susceptible streptococci (9, 10, 47). In the *S. equi* strains carrying the *zooA* gene, the gene is always accompanied by a neighboring immunity gene termed *zif*. The *zif* gene product protects the zoocin A producer strain by inserting an extra alanine residue in the peptidoglycan cross bridges of its cell wall (11). There is no *zif* immunity gene flanking the *S. agalactiae zooA* gene, suggesting that the two zoocin A homologues carry out different biological functions in their respective species.

Some investigators might argue that fratricins in reality are bacteriocins that are used by competent cells to eliminate competitors from the common habitat and that the release of transforming DNA from susceptible cells is only a side effect of this chemical warfare. The overwhelming majority of bacteriocins produced by low-GC Gram-positive bacteria are small peptides that have a narrow spectrum of activity. In general, these peptide bacteriocins exert their antibacterial effect by making pores in the cytoplasmic membrane of sensitive bacteria, resulting in leakage of low-molecular-weight cytoplasmic components, destruction of the proton motive force (PMF), and loss of viability (35). In some cases, however, it has been demonstrated that the bactericidal effect of peptide bacteriocins triggers the autolytic system of target bacteria (33). In these cases cell death is followed by cell lysis. In comparison to the ubiquitous peptide bacteriocins, few examples of muralytic antimicrobials are known. Among streptococci, zoocin A, millericin B, and stellalysin, produced by a few strains of *Streptococcus equi* subsp. *zooepidemicus*, *Streptococcus anginosus*, and *Streptococcus constellatus*, respectively, have been reported to be murein hydrolases that apparently serve as weapons to kill competing bacteria (2, 20, 47). In our view it is too simplistic to regard all bactericidal murein hydrolases solely as antimicrobial compounds. In all likelihood, some of them have evolved primarily for purposes related to their ability to release nutrients and/or DNA from target cells.

S. gordonii strain Challis seems to lack a competence-regulated self-protection mechanism corresponding to the ComM protein produced by *S. pneumoniae* (18). However, our DNA transfer experiments show that only a fraction of the cell population is lysed during cocultivation, demonstrating that most of the cells are somehow spared from the muralytic activity of LytF. This apparent immunity might be due to the operation of an undiscovered self-protection mechanism or could result from the experimental conditions used. It is, for example, possible that the external concentration of LytF reached in our *in vitro* cocultivation experiments was not sufficient to cause lysis of a substantial part of the cell population. Regardless of which explanation is correct, some mechanism must exist that controls the activity of LytF under natural conditions. If not, development of the competent state would be suicidal for *S. gordonii* cells.

The competence regulon of all species in the genus *Streptococcus* seems to include a murein hydrolase, implying that these cell wall-degrading enzymes must serve some important function associated with natural transformation. Based on previous studies in *S. pneumoniae*, we have proposed that the competence-induced murein hydrolase CbpD together with the immunity protein ComM constitutes a predatory mechanism used by competent pneumococci to acquire homologous DNA from noncompetent target cells sharing the same habitat (25). In the present study we have shown that LytF, a competence-regulated murein hydrolase produced by *S. gordonii* and at least 11 other streptococcal species, in most respects is a functional analogue of CbpD. This finding strengthens our hypothesis that the biological role of these murein hydrolases is to kill and lyse close relatives of the competent attacker cells in order to release homologous donor DNA. In a very recent report, Stevens et al. (49) show that increasing the error rate during ribosomal decoding promotes competence development in *S. pneumoniae*. This finding provides strong evidence that a major function of natural transformation in streptococci is repair of damaged DNA. Such repair will work only if competent streptococci possess a mechanism that enables them to capture DNA from closely related bacteria when growing in an environment consisting mostly of distantly related and unrelated bacteria, i.e., in multispecies biofilms in the oral cavity and nasopharynx. In our view, the best candidate for such a mechanism is the fratricide mechanism, which in the present study is shown to be omnipresent in the genus *Streptococcus*.

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