

Competence-Induced Cells of *Streptococcus pneumoniae* Lyse Competence-Deficient Cells of the Same Strain during Cocultivation

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Several streptococcal species are able to take up naked DNA from the environment and integrate it into their genomes by homologous recombination. This process is called natural transformation. In *Streptococcus pneumoniae* and related streptococcal species, competence for natural transformation is induced by a peptide pheromone through a quorum-sensing mechanism. Recently we showed that induction of the competent state initiates lysis and release of DNA from a subfraction of the bacterial population and that the efficiency of this process is influenced by cell density. Here we have further investigated the nature of this cell density-dependent release mechanism. Interestingly, we found that competence-induced pneumococci lysed competence-deficient cells of the same strain during cocultivation and that the efficiency of this heterolysis increased as the ratio of competent to noncompetent cells increased. Furthermore, our results indicate that the lysins made by competent pneumococci are not released into the growth medium. More likely, they are anchored to the surface of the competent cells by choline-binding domains and cause lysis of noncompetent pneumococci through cell-to-cell contact.

Natural transformation in streptococci can be divided into separate stages consisting of competence induction, double-stranded DNA binding, single-stranded DNA uptake, and homologous recombination (5, 11). In *Streptococcus pneumoniae* strain Rx, which has been used in this study, the process is regulated by a secreted 17-amino-acid-long peptide pheromone (CSP-1) which triggers competence development when it reaches an external concentration of 1 to 10 ng/ml (9). In a batch culture, this corresponds to about 10^7 cells/ml. CSP-1, which is encoded by *comC*, is secreted by an ABC transporter (ComAB) (12) and acts through a two-component regulatory system consisting of a histidine kinase receptor (ComD) and its cognate response regulator (ComE) (10, 18, 26). The alternative sigma factor ComX, which presumably is directly regulated by ComE, activates transcription of the late genes whose products are involved in DNA binding, DNA uptake, and recombination, etc. The late genes share a consensus promoter sequence called a cin-box (TACGAATA) (2, 19) that is recognized by ComX when this sigma factor is associated with the RNA polymerase holoenzyme (13, 15).

Although much has been learned about natural transformation in streptococci over the past decade, some important questions remain to be answered. One such question concerns the origin of the naked DNA taken up by competent cells, the so-called donor DNA. Traditionally it has been believed that DNA released from dead bacteria constitutes the only source of donor DNA and that donor DNA will always be present in the natural habitats of competent streptococci. In contrast to this view, evidence has recently been presented indicating that

a DNA release mechanism is operating during competence to ensure that donor DNA is available at the appropriate time (25). When a population of *S. pneumoniae* is induced to competence, a subfraction of the cell population will lyse and release its contents to the surroundings. DNA release and DNA uptake reach their maxima at about the same time, demonstrating that release of DNA from the donor cells is coordinated in time and space with uptake by the recipients (25). So far, the DNA release mechanism has not been fully elucidated, but it is known to be influenced by cell density and to be under control of the same quorum-sensing system that regulates competence development (ComCDE) (25). In addition, evidence has been obtained indicating that LytA and maybe additional choline-binding proteins (CBPs) are involved (25). In the present work, experiments were carried out to discern between autolysis and heterolysis (we define heterolysis as the lysis of one bacterium brought about by another). Our results show that competent pneumococci are capable of lysing noncompetent pneumococci of the same strain during cocultivation. These results suggest that heterolysis rather than autolysis is the mechanism of DNA release in a competent population of *S. pneumoniae*. However, it cannot be ruled out that both mechanisms operate simultaneously.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study were derived from *S. pneumoniae* strain Rx (Table 1). Bacterial strains were grown in casein tryptone (CAT) medium 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 (16). After sterilization, glucose was added to a concentration of 0.2%. Unless otherwise specified, all incubations were carried out at 37°C and all density measurements of bacterial cultures were done spectrophotometrically at 550 nm.

Construction of *S. pneumoniae* mutant strains. The H4 mutant was constructed by insertion-duplication mutagenesis. The primers LytA.1 (5'-ATATTGGTACCGCGTTGGAATGCTGAGACCTATGCAGC-3') and LytA.2 (5'-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
A1	CP1415 but Rif ^r through spontaneous conversion	This work
CP1200	Rx but <i>malM511 str-1</i>	24
CP1415	CP1200 but <i>comA</i> negative by insertion of the <i>ermAM</i> cassette into the <i>ClaI</i> site of <i>comA</i>	17
CP1500	<i>hex nov-r1 bry-r str-1 ery-r1 ery-r2</i>	3
EH1	EK4166 but <i>comE</i> negative by replacement with a Kan ^r cassette via double-crossover recombinations	This work
EK100	CP1415 but <i>egb</i> negative by transformation with genomic DNA from R262	25
EK4166	EK100 but <i>hirL::pEVP3</i> by transformation with plasmid DNA	25
EK4167	EK4166 but Nov ^r by transformation with CP1500 genomic DNA	25
H1	CP1415 but Nov ^r by transformation with CP1500 genomic DNA	25
H2	H1 but <i>comE::pXF518</i> by transformation with R319 genomic DNA	25
H3	H1 but <i>lytA::pEVP3</i> by transformation with plasmid DNA	25
H4	EK4166 but <i>lytA::pFW13</i> by transformation with plasmid DNA	This work
H5	EK100 but <i>lytC::pEVP3</i> by transformation with plasmid DNA	This work
H6	A1 but <i>LytA⁻</i> by transformation with genomic DNA from H3	This work
R262	R800 but negative for endogenous β -galactosidase activity (<i>ebg</i> negative)	1
R319	R800 but <i>comE::pXF518</i> by transformation with plasmid DNA	1
S1	CP1415 but <i>orf62-orf51::pEVP3</i> by transformation with plasmid DNA	This work
<i>S. pneumoniae</i> Rx	R36A derivative	21
Plasmids		
pEVP3	Nonreplicative vector (Cm ^r <i>lacZ</i>)	4
pFW13	Nonreplicative vector (Km ^r)	20

ATATTGGATCCGTACCAGTAGCCAGTGTCTTCTGCCC-3') were used to amplify a ~350-bp internal region of the *lytA* gene. Purified genomic DNA from strain CP1415 was used as a template. The resulting PCR fragment was cloned into the pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's recommendations. The DNA fragment was excised by *SpeI* and *ApaI* and ligated into multiple cloning site II (MCS-II) of the vector pFW13 (20), precleaved with the same enzymes. The ligation reaction was used to transform chemically competent TOP10 cells (Invitrogen), and positive clones were selected on agar plates containing 50 μ g of kanamycin/ml. The insertion-duplication mutant was made by natural transformation of EK4166 by using purified pFW13 containing the amplified 350-bp fragment. To verify that the correct mutant had been made, a PCR was performed using primer LYTA.3 (5'-GAA CAGATTTGCCTCAAGTCGGCG-3'), located upstream of primer LYTA.1, and primer pFW13.4 (5'-TAACCGTATTACCGCCTTTG-3'), located downstream of MCS-II in pFW13. A PCR product of the expected size was obtained, demonstrating that the pFW13 plasmid had integrated into the *lytA* gene. The H4 mutant also had the expected phenotype. It did not autolyse in the stationary phase even in the presence of 0.1% Triton X-100.

The H5 mutant was constructed in the same way as the H4 mutant. PCR was performed with the primers *LytC.1* (5'-TTAAGCATGCGAATGTCGGCTGG GTTCACAGAGATGG-3') and *LytC.2* (5'-TTAAGGATCCCTCAACATCAT AATAGATAGGGTAAGACAGG-3') containing *SphI* and *BamHI* sites at their 5' ends, respectively. The resulting ~380-bp internal fragment of the *lytC* gene was then cloned into the pCR2.1-TOPO vector. The cloned PCR fragment was excised from the pCR2.1-TOPO vector with *SphI* and *BamHI* and ligated into the pEVP3 vector (4), predigested with the same restriction enzymes. Finally, the H5 mutant was constructed by transforming competent EK100 cells with approximately 1 μ g of purified pEVP3 containing the 380-bp *lytC* fragment. Insertion-duplication mutants were selected on agar plates containing 2.5 μ g of chloramphenicol/ml. To confirm that the correct *LytC⁻* mutant had been made, control PCRs were carried out using genomic DNA from selected transformants as templates. *LytC.3* (5'-CAGATGGTCTGTTACTCGCA-GAATG-3'), corresponding to a sequence in the *lytC* gene upstream of the site of integration, was used in combination with pEVP3.3 (5'-GTAACCTCCACAGTAGTTCACCAC C-3') or pEVP3.4 (5'-CCCGTGCACCCGTAATCTTACGTC-3'). These primers are complementary to sequences in the pEVP3 vector. The resulting PCR fragments were of the expected sizes, demonstrating that the *lytC* gene had been disrupted and that pEVP3 had been inserted in the correct orientation, placing the promoterless *lacZ* gene of the pEVP3 vector under control of the *lytC* promoter.

Rimini et al. (23) have shown that the rate of transcription of two small open

reading frames termed *orf62* and *orf51* increases about 12-fold upon competence development. There is also a *cin*-box located immediately upstream of the start codon of *orf62*, showing that *orf62* and *orf51* belong to the ComX regulon. To be able to monitor expression of the late genes under various growth conditions, we wanted to construct a mutant containing a β -galactosidase reporter system controlled by the *orf62-orf51* promoter. This was carried out by amplifying a ~270-bp DNA fragment corresponding to about half of *orf62* and the complete *orf51* by PCR using the primers *orf62.1* (5'-CTCGCTCCCTGGTTATCTTTGGAG-3') and *orf51.2* (5'-CTAGCATGACTTACCAAACCTTTTACGAAGG-3'). The DNA fragment was first cloned into the pCR2.1-TOPO vector, then excised using the pCR2.1-TOPO polylinker restriction enzymes *BamHI* and *NsiI*, and finally ligated into the pEVP3 vector, precleaved with *BamHI* and *NsiI*. Purified pEVP3 vector harboring the 270-bp fragment was then used to transform the EK100 strain by natural transformation. Colonies growing on agar plates containing 2.5 μ g of chloramphenicol/ml were isolated and tested for β -galactosidase activity in the presence and absence of CSP-1. A mutant termed S1 that had the expected phenotype was isolated. It produced no β -galactosidase in the absence of CSP-1, but the addition of 250 ng of this peptide pheromone/ml induced strong expression of the β -galactosidase enzyme.

The EH1 strain is identical to EK4166, except that its *comE* gene is replaced by a Kan^r cassette via double-crossover recombinations. To make the EH1 strain, we took advantage of the pFW13 vector, which contains a kanamycin resistance gene flanked by two MCSs, MCS-I and MCS-II. An ~880-bp DNA fragment, corresponding to the region immediately upstream of the *comE* gene, was amplified by PCR using the primers *ComE.56* (5'-TTAAGCTAGCATCTTCGT TTCAGATATGGTAAGTACG-3') and *ComE.57* (5'-TTAAGACGTCCATCC AATATTCTCTCTAGTCTCACTTGATG-3') and ligated into the pCR2.1-TOPO vector as described above. The DNA fragment was excised by *AatII* and *NheI* and cloned into MCS-I of pFW13. Next, a ~750-bp DNA fragment, corresponding to the region immediately downstream of the *comE* gene, was amplified by PCR using the primers *comE.53* (5'-ATTACCATGGTCTCAAAAAG TGATTGACAATTAGCAAG-3') and *comE.55* (5'-ATTACATATGGCTATG GTACAATTACTGATGGAACAGCC-3') and cloned into the pCR2.1-TOPO vector. The fragment was excised from the pCR2.1-TOPO vector with *NcoI* and *NdeI* and ligated into MCS-II of the pFW13 vector harboring the 880-bp fragment in MCS-I. Using this construct as a template and *comE.55* and *comE.56* as primers, we amplified a ~3,500-bp fragment consisting of the Kan^r gene flanked with the two cloned fragments described above. This linear DNA fragment was then used to transform the EK4166 strain by natural transformation. A transformant growing on agar plates containing 150 μ g of kanamycin/ml was isolated and assayed for transformability by using genomic DNA from the novobiocin-

resistant strain CP1500. The transformant, termed EH1, turned out to be completely noncompetent, demonstrating that the Kan^r cassette most likely had replaced the *comE* gene. To further verify that this was the case, the site of integration of the Kan^r cassette was examined by PCR. The reaction was carried out with genomic DNA from EH1, the primer tArg2 (5'-CATAGCTCAGCTG GATAGAGCATTGCCTTC-3) that is complementary to the Arg-tRNA gene upstream of the *comCDE* operon, and the primer pFW13.6 (CATTTATTAC CTCCTTTT-GGTACCTCAC-3) that is complementary to the promoter region of the Kan^r gene. Analysis of the PCR by agarose gel electrophoresis revealed a single band of the expected size, demonstrating that the Kan^r cassette had integrated at the correct location.

β -galactosidase release assay. An overnight culture of the bacterial strain was diluted to an optical density at 550 nm (OD₅₅₀) of 0.1 in prewarmed (37°C) CAT medium and incubated until it reached an OD of 0.3. To ensure vigorous growth, it was rediluted to an OD₅₅₀ of 0.05 and incubated further. When the culture reached an OD of 0.1, two 10-ml samples were withdrawn and induced to competence by addition of 250 ng of CSP-1 (NH₂-EMRLSKFFRDFILQRKK-COOH)/ml, incubated for 30 min, and placed on ice. The cells in the first sample were immediately removed by centrifugation at 2,500 × *g* for 10 min at 4°C. Then the supernatant was sterile filtered using a 0.2- μ m-pore-size filter and stored on ice until assayed. In order to measure the total β -galactosidase activity in the culture, the cells in the second sample were lysed by incubating the culture for 10 min at 37°C with 0.1% Triton X-100. Following lysis, the culture was kept on ice until assayed. Samples were collected at regular intervals during logarithmic growth (OD₅₅₀s of 0.1 and 0.2, etc.). Uninduced samples were always run in parallel as negative controls. All samples were assayed for β -galactosidase activity as described previously (25). Cocultivation experiments were performed in the same way, except that 5 ml of each strain was mixed when the OD₅₅₀ of each culture reached 0.1 and 0.2, etc. In the experiment in which the effect of temperature on β -galactosidase release was examined, the cultures were placed at their respective temperatures 30 min before competence was induced by the addition of 250 ng of CSP-1/ml. At this stage, the OD₅₅₀s of the cultures had reached approximately 0.35. Apart from this, the assay was carried out as described above.

Cocultivation transformation assay. To measure the efficiency of gene exchange between two strains growing in liquid medium, a cocultivation assay was developed. Overnight cultures of the two strains H2 (ComA⁻ ComE⁻ Nov^r) and A1 (ComA⁻ Rif^r) were diluted to OD₅₅₀s of 0.05 and incubated at 37°C until they reached OD₅₅₀s of 0.2. To ensure vigorous growth, both cultures were diluted once more to OD₅₅₀s of 0.05 and incubated further at 37°C. When they reached OD₅₅₀s of 0.1, 0.2, and 0.4, 0.5 ml from each culture (H2 and A1) was withdrawn and mixed in a 15-ml plastic tube containing 250 ng of CSP-1. The mixed strains were incubated at 37°C for 30 min, diluted four times with prewarmed CAT medium, and then further incubated at 37°C for 60 min before being plating onto CAT agar plates containing 2 μ g of rifampin/ml and 5 μ g of novobiocin/ml. After incubation of the plates at 37°C for 24 to 48 h, the number of transformants resistant to both antibiotics was determined. Uninduced samples were run in parallel as negative controls. Identical gene exchange assays were carried out by cocultivating strains H3 and H6 and strains A1 and H1.

RESULTS

Competence-induced heterolysis. During competence development in a population of *S. pneumoniae* cells, a subfraction of the population was lysed, causing release of DNA to the surroundings (25). Addition of CSP-1 did not elicit a corresponding response in a competence-deficient mutant in which the response regulator ComE had been disrupted. ComE, which is part of the two-component regulatory system (ComDE) that monitors the external concentration of the competence pheromone, is a key component in the signal transduction pathway triggering competence development in *S. pneumoniae*. The absence of cell lysis in a ComE⁻ mutant clearly shows that the signal transduction pathway encoded by *comDE* also controls the expression of genes involved in cell lysis and strongly suggests that this phenomenon is an integral part of natural transformation (25).

To further elucidate the mechanism behind competence-induced cell lysis, we asked ourselves whether transfer of DNA

TABLE 2. Cocultivation of a competence-inducible strain (A1) and a competence-deficient strain at different densities during logarithmic growth

Deficient strain or DNA fragment	Pheromone added	No. of CFU of transformants/ml at OD ₅₅₀ of:		
		0.1	0.2	0.4
H2 ^a	Yes	1.8 × 10 ⁴	4.8 × 10 ⁴	9.7 × 10 ⁴
	No	2.0 × 10 ¹	0	1.0 × 10 ¹
Nov ^r DNA from CP1500 ^b	Yes	5.9 × 10 ⁵	3.4 × 10 ⁵	2.4 × 10 ⁵
	No	0	0	1.0 × 10 ¹

^a Competence-deficient mutant lacking a functional *comE* gene.

^b One microgram of purified DNA per milliliter from novobiocin-resistant strain CP1500 was used to transform strain A1.

can take place between a competence-inducible strain and a competence-deficient strain of *S. pneumoniae*. In order to answer this question, a cocultivation experiment was carried out with the two *S. pneumoniae* strains A1 (ComA⁻ Rif^r) and H2 (ComA⁻ ComE⁻ Nov^r). Vigorously growing cultures of A1 and H2 were mixed in equal amounts at OD₅₅₀s of 0.1, 0.2, and 0.4, subjected to 250 ng of CSP-1/ml, incubated for 1.5 h at 37°C, and spread onto agar plates containing 2 μ g of rifampin/ml and 5 μ g of novobiocin/ml (see Materials and Methods for details). The plates were incubated at 37°C, and the number of transformants resistant to both antibiotics was determined the next day. The results presented in Table 2 show that competence-induced A1 cells must have taken up DNA released from the competence-deficient H2 strain. In a negative control experiment run in parallel, CSP-1 was omitted. As expected, no transformant was obtained in this experiment. The Nov^r DNA taken up by the A1 cells may have originated from dead H2 cells and been present in the growth medium of the H2 cells before they were mixed with the A1 cells. Alternatively, release of Nov^r DNA may have been an active process mediated by the A1 cells after they had been induced to competence by addition of CSP-1. To discern between these two mechanisms, we set up a cocultivation experiment in which the competence-deficient (ComE⁻) strain EH1 expressing *Escherichia coli* β -galactosidase from a constitutive promoter was mixed with an equal amount of the competence-inducible strain EK100 lacking this enzyme. When the EK100 strain was induced to competence by addition of CSP-1, cytoplasmic β -galactosidase from the EH1 strain was released to the growth medium. However, very little β -galactosidase activity was detected in the medium of the negative control, i.e., when no CSP-1 had been added to the mixed culture (Fig. 1). Furthermore, when CSP-1 was added to a culture consisting only of the EH1 strain, no release of β -galactosidase was detected (results not shown). These results can only be explained by a release mechanism that involves heterolysis, i.e., lysis of the competence-deficient EH1 strain by the competent EK100 strain.

Do competent cells release a lysin? Previously it was shown that the fraction of cells lysed when cultures of *S. pneumoniae* are induced to competence at different cell densities increases with OD₅₅₀s of up to 0.3 to 0.4 and then declines (25). The mechanism behind this cell density dependency is not under-

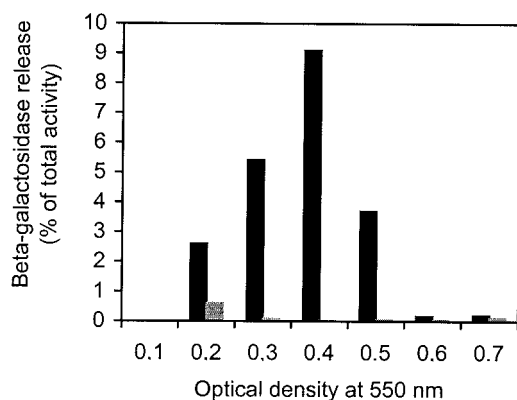


FIG. 1. Competence-induced release of intracellular β -galactosidase into the growth medium during cocultivation of *S. pneumoniae* strains EH1 and EK100. The EH1 strain is competence deficient and expresses β -galactosidase from a constitutive promoter. In contrast, the EK100 strain is competence inducible but β -galactosidase deficient. Cultures of EH1 and EK100 growing in parallel were mixed at a ratio of 1:1 when they reached OD_{550} s of 0.1 and 0.2, etc. After 30 min with or without CSP-1, samples were collected for analysis of β -galactosidase activity. Both cell-free growth medium (supernatants) and cell lysates (supernatants and lysed cells) were assayed. The amount of β -galactosidase present in the supernatants (estimated in Miller units) is given as a percentage of the total activity present in the cell lysates. Black columns represent cocultivation in the presence of 250 ng of CSP-1/ml. Grey columns represent cocultivation in the absence of CSP-1. The results shown are representative of results from three separate experiments.

stood, but it is likely that the decline in release can be explained, at least in part, by loss of competence induction towards the end of the logarithmic growth phase (9). Our finding that competent cells can lyse competence-deficient cells suggests that competent cells may secrete a lysin. If this is the case, it would be expected that increasing the ratio of competence-inducible cells to competence-deficient cells would result in more-efficient lysis. In cocultivation experiments similar to the one described above, different ratios of strains EH1 and EK100 were used (Fig. 2). The results of these experiments clearly showed that the percentage of EH1 cells lysed increased when the ratio of competent EK100 cells to competence-deficient EH1 cells increased. Thus, the competent cells must produce one or more lysins that attack noncompetent cells and lyse them. If the lysin(s) is secreted into the external medium, it should be possible to induce lysis of noncompetent cells by adding cell-free medium from a culture of competent cells. A vigorously growing culture of EK100 was induced to competence by addition of 250 ng of CSP-1/ml at an OD_{550} of 0.4 and incubated at 37°C for 30 min. The cells were removed by centrifugation, and the supernatants were sterile filtered. Uninduced samples were run in parallel as negative controls. The temperature of the sterile-filtered supernatants was adjusted to 37°C, and then the supernatants were mixed immediately with equal volumes of vigorously growing cultures of EH1 cells. These mixtures were incubated for another 30 min, and the supernatants were harvested and assayed for β -galactosidase activity as described before. We did not detect any release of β -galactosidase into the culture sample in which EH1 cells had been mixed with growth medium from competence-induced

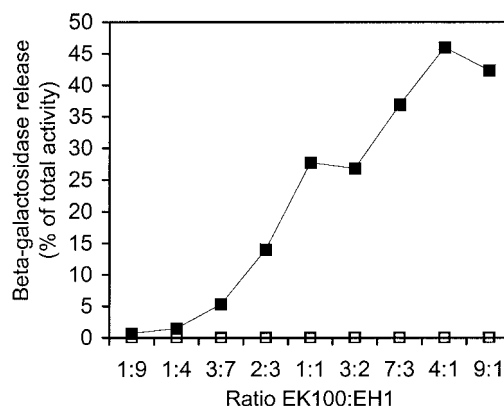


FIG. 2. Release of cytoplasmic β -galactosidase from the competence-deficient EH1 strain during cocultivation with strain EK100. The EK100 strain is competence inducible when CSP-1 is added but is completely without endogenous β -galactosidase activity. When cultures of the two strains both reached an OD_{550} of 0.4, they were mixed at different ratios and 250 ng of CSP-1/ml was added immediately. After 30 min, samples were harvested and cell-free supernatants and cell lysates (supernatants and lysed cells) were prepared and assayed for β -galactosidase activity. Uninduced samples were run in parallel as negative controls. The amount of β -galactosidase present in the supernatants (estimated in Miller units) is given as a percentage of the total activity present in the cell lysates. Closed squares represent results for supernatants from CSP-1-induced cells, and open squares represent results for supernatants from uninduced cells. The experiment was repeated several times with similar results.

EK100 cells (results not shown). To ensure that the EH1 cells employed in these experiments produced normal amounts of β -galactosidase, parallel samples were lysed by addition of 0.1% Triton X-100 and their total enzyme contents were measured. The levels of total β -galactosidase activity present in these samples were as expected. In conclusion, it seems that the lysin produced by competent *S. pneumoniae* cells is not released into the growth medium. Rather, our results suggest that the lysin is attached to the cell surface and that cell-cell contact is necessary for heterolysis to take place.

Role of LytA. A good candidate for the unidentified lysin discussed above is the murein hydrolase LytA, which is anchored noncovalently to the pneumococcal cell wall through six choline-binding domains (14). It is expressed in noncompetent cells, but its expression is upregulated about sixfold during competence development (23). It has previously been shown that a $LytA^-$ mutant releases less DNA when induced to competence than a wild-type strain (25). Here we decided to make a $LytA^-$ deficient mutant that expresses *E. coli* β -galactosidase constitutively (H4) and to measure CSP-1-induced release of β -galactosidase from this mutant at different cell densities. It turned out that significantly less β -galactosidase is released from the $LytA^-$ mutant during competence development than from the positive control. The difference is two- to fourfold depending on the cell density of the culture (Fig. 3). However, at OD_{550} s of 0.3 and 0.4, the amount of β -galactosidase released from the $LytA^-$ mutant is still almost 10-fold higher than that released from the uninduced control (Fig. 3). This indicates that more than one lysin is involved in the release mechanism. This view is supported by the results of the DNA exchange experiment presented in Fig. 4. Strains resis-

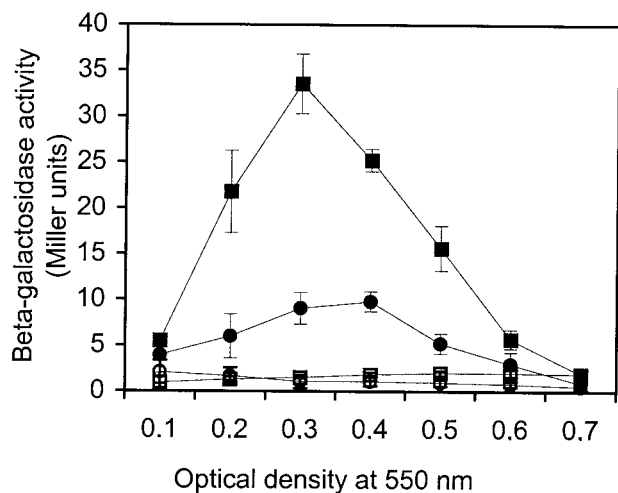


FIG. 3. Comparison of strains EK4166 and H4 with respect to the level of competence-induced release of cytoplasmic β -galactosidase. The *lytA* gene of strain H4 has been disrupted, but otherwise the strain is identical to EK4166. At different cell densities (OD_{550} s of 0.1 to 0.7), samples were withdrawn and induced to competence by adding 250 ng of synthetic CSP-1/ml. After 30 min at 37°C, cell-free supernatants were prepared and assayed for β -galactosidase activity. Corresponding uninduced samples were run in parallel as negative controls. Closed squares, activity in supernatants from CSP-1-induced EK4166 cells; open squares, activity in supernatants from uninduced EK4166 cells; closed circles, activity in supernatants from CSP-1-induced H4 cells; open circles, activity in supernatants from uninduced H4 cells. The β -galactosidase activity is given in Miller units, and amounts shown are the means \pm standard errors of results for triplicate samples.

tant to novobiocin (H1) and rifampin (A1) were cocultivated at OD_{550} s of 0.1, 0.2, and 0.4 as described above, and transformants were selected on agar plates containing 2 μ g of rifampin/ml and 5 μ g of novobiocin/ml. In parallel, the two *LytA*⁻ mutants H3 and H6, resistant to novobiocin and rifampin, respectively, were cocultivated and the number of transformants resistant to both antibiotics was determined. The results show that the numbers of transformants obtained with the *LytA*⁻ mutants are four- to eightfold lower (depending on cell density) than the corresponding numbers obtained with the positive control.

LytC is not the missing lysin. Evidently, *LytA* plays an important role in competence-induced DNA release in *S. pneumoniae*. However, as demonstrated above, there must be at least one additional lysin produced by competent pneumococci. The release mechanism is sensitive to the presence of choline in the growth medium, demonstrating that one or more CBPs are involved (25). Without affecting transformability, the presence of 0.02% choline reduces release of β -galactosidase by about 50% and 0.2% reduces release by up to 90% (results not shown). We therefore hypothesized that another choline-binding murein hydrolase could be involved. The most promising candidate was the choline-binding lysozyme *LytC* (7, 14), which had been reported to be competence induced (23). *LytC* has optimum activity at 30°C (7), and we therefore first investigated whether the competence-induced lysis mechanism is more efficient at this temperature. The results given in Fig. 5 show that this indeed is the case. Release of β -galactosidase at 30°C increases two- to threefold compared to the correspond-

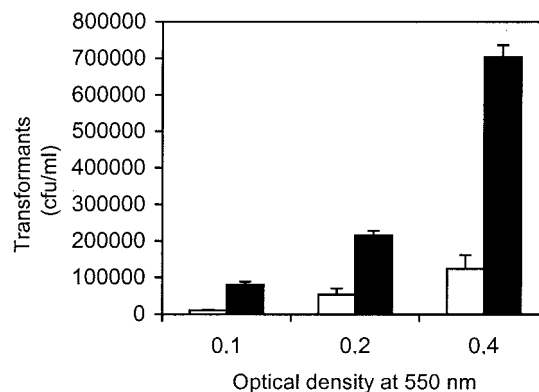


FIG. 4. Influence of *LytA* on efficiency of gene exchange in vitro. Two *LytA*-deficient *S. pneumoniae* strains, H3 (Nov^r) and H6 (Rif^r), were mixed at three different densities (OD_{550} s of 0.1, 0.2, and 0.4) and induced to competence by adding 250 ng of CSP-1/ml. After cocultivation for 1.5 h, cells were plated onto agar plates containing rifampin and novobiocin and incubated at 37°C. Transformants were scored the next day. The two strains H1 (Nov^r) and A1 (Rif^r), both containing intact *lytA* genes, were treated in the same way. Black columns represent numbers of transformants obtained by cocultivation of H1 and A1, while white columns represent numbers of transformants obtained by cocultivation of the *LytA*-deficient strains H3 and H6. Values shown are numbers of CFU of transformants per milliliter and are the means \pm standard errors of results for triplicate samples. Uninduced samples, receiving no peptide pheromone, were run in parallel as negative controls. The numbers of transformants observed in uninduced samples ranged from 0 to 200 CFU/ml.

ing release at 37°C. Interestingly, we also found that release is practically abolished at 40°C. In principle, high temperatures may influence release of β -galactosidase indirectly by suppressing competence induction or they may act directly on the cell lysis mechanism. In order to address this question, we used a mutant (S1) that harbors a chromosomally located *lacZ* re-

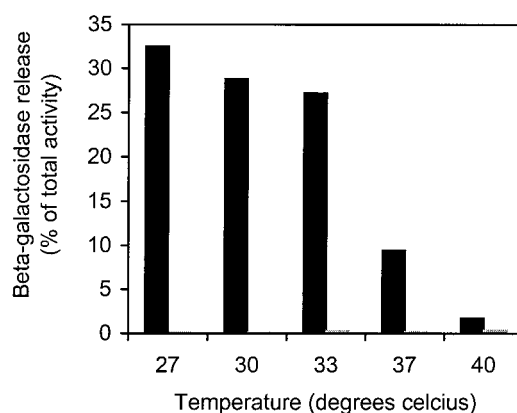


FIG. 5. Effect of temperature on competence-induced release of cytoplasmic β -galactosidase from *S. pneumoniae*. Strain EK4166 was induced to competence at five different temperatures by addition of 250 ng of CSP-1/ml (black columns). Both cell-free supernatants and cell lysates (supernatants and lysed cells) were assayed for β -galactosidase activity. The amount of β -galactosidase present in the supernatants (estimated in Miller units) is given as a percentage of the total activity present in the cell lysates. Corresponding uninduced samples were run in parallel as negative controls (grey columns). This experiment has been repeated several times with similar results.

porter gene under control of the *orf62-orf51* promoter (23). *orf62* and *orf51* are two small bacteriocin-like reading frames that are part of the ComX regulon. Two cultures of the S1 strain ($OD_{550} = 0.35$) growing at 37 and 40°C were induced to competence by addition of CSP-1, incubated for 30 min, lysed by addition of 0.1% (vol/vol) Triton X-100, and assayed for β -galactosidase activity. Uninduced samples were run in parallel. No β -galactosidase activity was detected in the negative controls (results not shown). The activity measured in the induced S1 culture grown at 40°C (153 Miller units) was somewhat reduced compared to the activity measured in the corresponding culture grown at 37°C (207 Miller units). However, the reduction does not seem large enough to explain the nearly complete loss of competence-induced cell lysis at 40°C. It must therefore be concluded that the observed effect is due to temperature sensitivity of the release mechanism itself.

Together, the various data discussed above suggested to us that LytC could be involved in competence-induced cell lysis. We therefore decided to make a *lytC* knockout mutant. An internal fragment of *lytC* was cloned into the pEVP3 vector, and this construct was used to transform strain EK100. The pEVP3 vector contains a promoterless *E. coli lacZ* reporter gene immediately downstream of the polylinker. Transforming strain EK100 with this construct resulted in integration of the recombinant plasmid into the *lytC* gene by an insertion-duplication mechanism, giving rise to a chromosomally located transcriptional fusion between *lytC* and *lacZ*. The strain (H5) carrying this reporter construct was used to control the results of a previously published microarray analysis indicating that transcription of *lytC* is upregulated during competence development (23). Unexpectedly, our results showed that rates of transcription from the *lytC* promoter remained the same in induced and uninduced cultures, demonstrating that *lytC* is not a competence-regulated gene. However, as the *lacZ* gene was constitutively transcribed at a high rate, the H5 strain could be used directly in a β -galactosidase release assay. The results of this assay (Fig. 6) showed that the level of β -galactosidase released into the growth medium when the LytC⁻ mutant was induced to competence was not reduced compared to the level released by the wild type. We therefore concluded that LytC is not involved in the competence-activated lysis mechanism.

DISCUSSION

The data presented here and in a recent paper by Steinmoen et al. (25) clearly show that *S. pneumoniae* cells grown under laboratory conditions actively release DNA when induced to competence. This coordination between DNA release and uptake makes efficient gene exchange possible in a batch culture, as demonstrated by our cocultivation experiments with Nov^r and Rif^r mutants of *S. pneumoniae* strain Rx (Table 2 and Fig. 4). Since both DNA uptake and release are controlled by the same quorum-sensing system (ComABCDE), these processes must be coordinated also under natural conditions. From an evolutionary perspective, this finding adds a new dimension to natural genetic transformation and makes this phenomenon a biologically significant event. In order to better understand how gene exchange takes place under natural conditions, it is essential to elucidate fully the DNA release mechanism. All evidence gathered so far indicates that DNA is released by cell

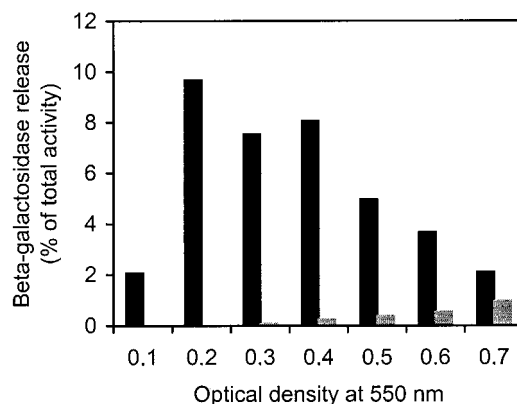


FIG. 6. Competence-induced release of β -galactosidase from the LytC-deficient H5 strain during logarithmic growth. Samples were collected at different cell densities (OD_{550} s of 0.1 to 0.7) and induced to competence by adding 250 ng of CSP-1/ml (black columns). Both cell-free supernatants and cell lysates (cells and supernatants) were assayed for β -galactosidase activity. The amount of β -galactosidase present in the supernatants (estimated in Miller units) is given as a percentage of the total activity present in the cell lysates. Corresponding uninduced samples were run in parallel as negative controls (grey columns). The presented data are representative of results from three independent experiments.

lysis and not by DNA export machinery. In the present work, we wanted to establish that release of DNA in a competence-induced population of *S. pneumoniae* takes place by autolysis. However, to our surprise we found that competence-deficient cells (ComE⁻) are lysed when cocultivated with competence-induced cells, demonstrating that heterolysis must take place. This finding does not exclude autolysis, but the high levels of β -galactosidase released in the experiments whose results are presented in Fig. 1 and 2 suggest that heterolysis contributes substantially to the process. This made us wonder whether the observed heterolysis is due to secretion of a lysin by the competent cells or whether it depends on cell-to-cell contact between competent and competence-deficient cells. We were unable to detect an active agent in the supernatant harvested from competence-induced *S. pneumoniae* cultures, indicating that heterolysis involves lytic substances attached to the surface of competent cells. This model is in agreement with the fact that in most experiments release is more efficient at OD_{550} s of 0.3 to 0.4 than at lower cell densities. In a batch culture, cell-to-cell contact will be more extensive at higher cell densities, leading to more-efficient lysis. The drop in release seen at even higher cell densities (OD_{550} s of 0.6 to 0.7) is probably due to the loss of competence inducibility (insensitivity to CSP-1) seen at high cell densities (9). Our results show that the presence of 0.2% choline in the growth medium almost completely abolishes competence-induced lysis without affecting transformability. Previous studies have demonstrated that CBPs can be eluted from the pneumococcal surface with soluble choline (6, 8). This suggested that a cell wall-located CBP is involved in the observed lysis (25). The obvious candidate for this role was the competence-induced amidase LytA, which is anchored to the cell wall through a choline-binding domain consisting of six repeats. It turned out that DNA release in a LytA⁻ mutant is significantly reduced, but not abolished, compared to that in

the wild type (25). This finding was confirmed by the results of the β -galactosidase release assay shown in Fig. 3. Consequently, an additional choline-binding murein hydrolase seems to be involved. The pneumococcal CBP family consists of 12 members, which include the three murein hydrolases LytA, LytB, and LytC (8, 14). LytB is involved in the separation of daughter cells (6), whereas the exact biological role of LytC is unknown. However, LytC possesses lysozyme-like activity with a temperature optimum of 30°C (6, 7). In addition, it has been reported that transcription of the *lytC* gene is upregulated during competence development (23). To determine whether LytC could be the missing lysin, we cocultivated competent and competence-deficient strains at different temperatures. The results (Fig. 5) showed that lysis is more efficient at temperatures around the reported optimum temperature for LytC. To further investigate the possible role of LytC in competence-induced cell lysis, we constructed a LytC knockout mutant. In this mutant, a *lacZ* reporter gene was placed under control of the *lytC* promoter. In contrast to the report by Rimini et al. (23), we found that the *lytC* gene was constitutively highly expressed, but its rate of transcription did not increase during competence development. In addition, no reduction in the release of β -galactosidase was detected (Fig. 6), demonstrating that LytC is not involved in competence-induced cell lysis.

Unexpectedly, the lysis mechanism works very poorly, if at all, at 40°C (Fig. 5). Our results indicate that the temperature-sensitive step is downstream of *comX*, since transcriptional activation of the late genes functions normally at 40°C. Presumably, a late-gene product which constitutes a critical part of the lysis machinery does not function at this temperature. Another possibility is that Brownian movements, which are more intense at 40°C, disrupt stable cell-to-cell contact, preventing heterolysis.

Most likely, gene exchange under natural conditions takes place in complex multispecies biofilms, where there is close contact between individual cells. In oral streptococci such as *S. mitis* and *S. gordonii*, this contact is mediated by surface-exposed proteins called coaggregation adhesins (22). Presumably, pneumococci rely on similar surface proteins to make contact with one another and other species in the biofilm community. To better understand how gene exchange takes place under natural conditions, it is important to understand exactly how DNA is released from the donor cells and how this process is regulated. In a batch culture, competence is triggered when the concentration of CSP-1 reaches 1 to 10 ng/ml, corresponding to about 10⁷ cells/ml. Within a biofilm community, diffusion of the CSP-1 peptide is probably much more restricted and competence most likely develops among small clusters of cells belonging to the same phenotype. Our results suggest that competent cells in such clusters can lyse neighboring cells by expressing cell wall-degrading enzymes such as LytA on their surfaces. We therefore postulate that competent streptococci actively acquire DNA by killing their neighbors. This contrasts with the traditional view in which it is assumed that DNA taken up by competent bacteria originates from donor bacteria that have died and fallen apart from natural causes.

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