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Natural transformation in *Streptococcus thermophilus:* Regulation, autolysis and ComS*- controlled gene expression

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TABLE OF CONTENTS

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SUMMARY

Natural genetic transformation has been extensively studied in *Streptococcus pneumoniae* for generations, and much has been learned about this important phenomenon since its discovery by Frederick Griffith in 1928. In comparison, natural transformation has been little studied in other streptococcal species. The majority of streptococci have, in fact, never been observed to be naturally transformable. *Streptococcus thermophilus* has traditionally been considered to be a non-competent species. It is widely used by the dairy industry in the production of a variety of food products, and is consequently of great economic importance. To further improve the properties of *S. thermophilus* as a dairy starter, a better understanding of its genetics, metabolism and physiology is essential. Progress in this area has been hampered by the lack of efficient genetic tools. Some years ago, Blomqvist and co-workers therefore set out to investigate whether *S. thermophilus* could be made competent for natural transformation by artificial overexpression of the alternative sigma factor ComX. This strategy proved successful resulting in a new tool that opened up new possibilities with respect to genetic manipulation of the *S. thermophilus* genome. Furthermore, the discovery sparked the interest of a number of research groups, leading to increased activity in this field.

In the current study, an important goal was to better understand how expression of ComX, the master switch of competence induction in streptococci, is regulated in *S. thermophilus*. Our results show that ClpC, presumably in complex with ClpP, regulates the level of ComX in the bacterial cell post-transcriptionally. ClpC is not part of the quorum-sensing-like competence-induction pathway recently identified by Fontaine and colleagues, but seems to be part of a control mechanism that prevents spontaneous competence induction in *S. thermophilus* under conditions that are sub-optimal or inappropriate for competence development (Paper I).

In *S. pneumoniae* competent cells produce and secrete a murein hydrolase (CbpD-Sp) that kills and lyses non-competent pneumococci and members of related species. Evidence strongly indicates that the biological function of CbpD-Sp is to mediate release of homologous DNA from target cells that can be taken up by competent cells to serve as templates for recombinational DNA repair. *In silico* screening of the *S. thermophilus* genome showed that it encodes a CbpD-like protein (CbpD-St) with a unique C-terminal domain. Consequently, it was of interest to determine whether this protein carries out the same function in *S. thermophilus* as in *S. pneumoniae*. Our results showed that the properties of

CbpD-St and CbpD-Sp are similar in most respects. Both proteins are murein hydrolases that bind to the surface of their respective host cells via their C-terminal domains. Both proteins also have the potential to lyse susceptible cells. However, in contrast to its pneumococcal counterpart, CbpD-St has a positive effect on the transformability of *S. thermophilus* (paper II). Thus, although our results indicate that CbpD-St and CbpD-Sp probably are functional analogues, it cannot be ruled out that CbpD-St serves a different biological function in *S. thermophilus*.

While the present study was in progress, the sought-after quorum-sensing pathway controlling competence induction in *S. thermophilus* was identified by Fontaine *et al.* The pathway turned out to be completely unrelated to the corresponding quorum-sensing pathway in *S. pneumoniae*. This made us realize that the *S. thermophilus* pathway, in principle, could be developed into a peptide-regulated gene depletion system for use in *S. pneumoniae*. The pathway consists of a signalling peptide (ComS*), which is imported into the cytoplasm by the Ami oligopeptide transporter, and an intracellular transcriptional activator termed ComR. According to the model proposed by Fontaine *et al.,* ComR becomes activated upon binding to ComS*. In the active state ComR induces expression of ComX by binding to the *comX* promoter. By introducing the *comR* gene and *comX* promoter fused to the gene of interest into the genome of *S. pneumoniae* we were able to show that the gene depletion system functioned as intended. The essential *licD1*gene, which is required for the synthesis of wall and lipoteichoic acids in *S. pneumoniae*, was used as a test case. Our results showed that depletion of the *licD1* gene gives rise to oversized, elongated and misshapen cells, indicating that pneumococcal cells with low levels of teichoic acids struggle to divide. In sum, the ComRS-based gene depletion system described in paper III has excellent properties that should make it a very useful tool for the study of essential genes in *S. pneumoniae* and other Gram-positive bacteria.

SAMMENDRAG

Naturlig genetisk transformasjon hos *Streptococcus pneumoniae* har vært gjenstand for omfattende studier i generasjoner. I årene som har gått siden Fredrick Griffith oppdaget naturlig transformasjon i 1928, har vi derfor lært mye om dette viktige fenomenet. Til sammenligning har naturlig transformasjon vært lite studert hos andre streptokokkarter. Prosessen har faktisk aldri vært observert hos de fleste artene i slekten *Streptococcus*. Tradisjonelt sett har det vært antatt at *Streptococcus thermophilus* mangler denne egenskapen, dvs. at den er en ikke-transformerbar art. *S. thermophilus* er mye brukt til fermentering av ulike meieriprodukter, og har derfor svært stor økonomisk betydning. For å forbedre egenskapene til *S. thermophilus* ytterligere trengs det mer kunnskap og en bedre forståelse av bakterien's genetikk, metabolisme og fysiologi. Mangelen på et effektivt genetisk verktøy har vært en bremsekloss for utviklingen på dette området. Det var grunnen til at Blomqvist og medarbeidere for en del år siden undersøkte om "kunstig" overuttrykking av den alternative sigma faktoren ComX kunne indusere kompetanse for naturlig transformasjon hos *S. thermophilus*. Denne strategien viste seg å være vellykket, noe som resulterte i et nytt kraftig genetisk verktøy for denne melkesyrebakterien. I tillegg resulterte denne oppdagelsen i økt forskningsaktivitet ved at flere forskningsgrupper ble interessert i dette feltet.

Et viktig mål for dette doktorgradsarbeidet var å få en bedre forståelse av hvordan ekspresjonen av ComX, som er en slags "hovedbryter" for kompetanseinduksjon hos streptokokker, reguleres hos *S. thermophilus*. Våre resultater viste at ClpC, sannsynligvis i kompleks med ClpP, regulerer konsentrasjonen av ComX i bakteriecellen post-transkripsjonelt. Fontaine *et al.* (2010) har nylig identifisert en "quorum-sensing"-lignende signal-overføringsvei som induserer ekspresjon av ComX og dermed kompetanse hos *S. thermophilus*. ClpC er ikke en del av denne veien, men ser ut til å være en del av en mekanisme som forhindrer at spontan kompetanseinduksjon hos *S. thermophilus* finner sted under forhold som er suboptimale eller ugunstige for naturlig transformasjon (Artikkel I).

Pneumokokker som er kompetente for naturlig transformasjon produserer og skiller ut en murein hydrolase (CbpD-Sp) som dreper og lyserer ikke-kompetente pneumokokker og medlemmer av beslektede arter. Eksperimentelle bevis indikerer at den biologiske funksjonen til CbpD-Sp er å frigjøre homologt DNA som deretter kan tas opp av kompetente celler og fungere som templat for DNA reparasjon. Søk i databaser viste at genomet til *S. thermophilus* koder for et CbpD-lignende protein (CbpD-St) med et unikt C-terminalt domene. Dette vakte

vår interesse, og vi bestemte oss for å forsøke finne ut om dette proteinet har samme funksjon hos *S. thermophilus* som CbpD-Sp har hos *S. pneumoniae*. Resultatene viste at CbpD-St og CbpD-Sp har mange like eller lignende egenskaper. Begge er murein hydrolaser som binder til overflaten av sine respektive vertsceller via sine C-terminale domener. I tillegg er begge i stand til å forårsake lysis hos følsomme celler. Vi oppdaget imidlertid en viktig forskjell på de to murein hydrolasene. I motsetning til CbpD-Sp øker tilstedeværelsen av CbpD-St transformasjonseffektiviteten hos *S. thermophilus* (Artikkel II). Til sammen tyder våre resultater på at CbpD-Sp og Cbp-St er funksjonelle analoger som begge er involvert i frigjøring av såkalt donor-DNA. På den annen side kan vi heller ikke utelukke at CbpD-St og CbpD-Sp utfører ulike funksjoner hos sine respektive arter.

Mens det foreliggende studiet pågikk ble den ovenfor nevnte ”quorum-sensing”-lignende signaloverføringsveien som induserer kompetanse hos *S. thermophilus* identifisert av Fontaine *et al.*. Det viste seg at denne signaloverføringsveien er totalt ubeslektet med den tidligere identifiserte signaloverføringsveien som benyttes til kompetanseinduksjon hos *S. pneumoniae*. Dette gav oss ideen til å forsøke å konstruere et titrerbart peptid-kontrollert genekspresjonssystem til studier av essensielle gener hos pneumokokker. Med et slikt system ville vi kunne uttrykke essensielle gener ektopisk, dvs. via det peptid-kontrollerte genekspresjonssystemet fra *S. thermophilus*. Etter delesjon av det native målgenet vil så den ektopiske transkripsjonen kunne senkes gradvis til fenotypiske trekk som gir informasjon om funksjonen til det essensielle genet manifesterer seg. Signaloverføringsveien som ble identifisert av Fontaine *et al*. består av et signalpeptid (ComS*), som transporteres inn i cytoplasmaet av en oligopeptidtransporter kalt Ami, pluss en intracellulær transkripsjons-aktivator kalt ComR. Ifølge den foreslåtte modellen aktiveres ComR når denne aktivatoren binder til ComS*. I aktiv tilstand induserer ComR ekspresjonen av ComX ved å binde til *comX* promoteren. Ved å integrere *comR* genet og *comX* promoteren fusjonert til målgenet i genomet til *S. pneumoniae* ble vi i stand til å vise at systemet vårt fungerte etter hensikten. Vi brukte det essensielle *licD1* genet, som er nødvendig for syntesen av teikoinsyre hos pneumokokker, til å prøve ut systemet vårt på et reelt biologisk problem. Resultatene viste at en gradvis reduksjon i mengden av LicD1 gav opphav til celler som var mye større og lengre enn normalt, noe som indikerer at pneumokokker med for lite teikoinsyre får problemer med å dele seg. Dette vellykkede resultatet viser at det titrerbare ekspresjonssystemet vi utviklet i artikkel III fungerer utmerket, og at det følgelig burde kunne bli et nyttig verktøy for studier av essensielle gener i pneumokokker og andre Gram-positive bakterier.

LIST OF PAPERS

List of papers included in this thesis:

Paper I:

Biørnstad, T.J. & Håvarstein, L.S. (2011) ClpC acts as a negative regulator of competence in *Streptococcus thermophilus*. *Microbiology*, 157, 1676-1684.

Paper II:

Biørnstad, T.J., Ohnstad H.S. & Håvarstein L.S. (2011) Deletion of the murein hydrolase CbpD reduces transformation efficiency in *Streptococcus thermophilus. Microbiology*, (Submitted manuscript).

Paper III:

Berg, K.H., Biørnstad, T.J., Straume, D., Håvarstein, L.S. (2011) Peptide-regulated gene depletion system developed for use in *Streptococcus pneumoniae. Journal of Bacteriology,* 193(19):5207-15.

1. INTRODUCTION

1.1 The genus *Streptococcus*

1.1.1 Taxonomy of streptococci

Members of the genus *Streptococcus* belong to the family *Streptococcaceae*, a part of the order *Lactobacillales*. Other well known genera in this order are *Lactobacillus* and *Enterococcus*. The order *Lactobacillales* is placed in the class Bacilli, which belongs to the phylum Firmicutes. Previously this phylum included all Gram-positive bacteria, however, recently the Gram-positives have been split into two separate phyla; the Firmicutes (low G+C Gram-positive bacteria) and the Actinobacteria (high G+C Gram-positive bacteria). Since the first characterisation of the genus *Streptococcus*, it has been revised many times. Subgroups have been transferred to new genera, single species have been redefined or removed and a number of new species have been added (Kilian, 1998).

Early classification of streptococci was based on their hemolytic properties when grown on blood agar, i.e. α-hemolysis, β-hemolysis and γ-hemolysis. α-hemolysis, which appears as green zones around colonies, is due to oxidation of the hemoglobin by bacterially produced H_2O_2. β-hemolysis appears as clear zones surrounding the colonies, and is due to lysis of the red blood cells by secreted hemolysins. Streptococci that display no zones or haloes around their colonies are called γ-hemolytic (Kilian, 1998). Traditionally *Streptococcus thermophilus* has been classified as γ-hemolytic (Sherman *et al.*, 1931), but in our hands *S. thermophilus* is clearly α-hemolytic.

Today's classification relies more on molecular methods, and has in the last 20 years mainly been based on DNA-DNA hybridization and/or 16S rDNA gene sequencing (Facklam, 2002). MLST (Multilocus Sequence Typing) and whole genome sequencing has also played an increasingly prominent role in the determination of taxonomic and phylogenetic relationships (Enright *et al.*, 1999). Based on the 16S rDNA sequences of 34 different streptococcal species, Kawamura *et al.* (1995) divided streptococci into six phylogenetic groups, a classification still used today (Kawamura *et al.*, 1995) (Fig. 1). The pyogenic group contains mostly β-hemolytic species, many of which are human and animal pathogens. Members of the anginosus and mitis groups are predominantly commensal species inhabiting the oral cavity and the intestinal tract. However, several species in these groups are opportunistic pathogens that may cause disease in for instance immunocompromised patients.

The mitis group also includes *Streptococcus pneumoniae*, an important pathogen that is the etiological agent of pneumonia, otitis media and meningitis in humans (Hardie *et al.*, 1997). *S. thermophilus*, which is a domesticated species used by the dairy industries in food fermentations, belongs to the salivarius group. The closest naturally occurring relatives of *S. thermophilus* are *Streptococcus salivarius* and *Streptococcus vestibularis*, which are found in the human oral cavity and upper respiratory tract. This suggests that the ancestor of *S. thermophilus* was part of the human oral flora similar to its closest relatives (Facklam, 2002). Recently, however, *S. thermophilus* was isolated from plants in Bulgaria, and consequently it has been proposed that the natural habitat of this species is plants (Michaylova *et al.*, 2007). The taxonomic status of *S. thermophilus* has been the subject of much debate, and for a period it was classified as *S. salivarius* ssp. *thermophilus.* In 1991, however, Schleifer *et al.* finally settled the dispute by providing evidence based on DNA-DNA hybridization that justified a full species status for *S. thermophilus* (Schleifer *et al.*, 1991). The bovis group contains species that are commonly found in the normal gut flora of ruminants (*Streptococcus bovis*) and horses (*Streptococcus equinus*). *Streptococcus mutans* and *Streptococcus sobrinus*, which belong to the mutans group, are both human pathogens that enhance the formation of caries within teeth (Hardie *et al.*, 1997; Kilian, 1998).

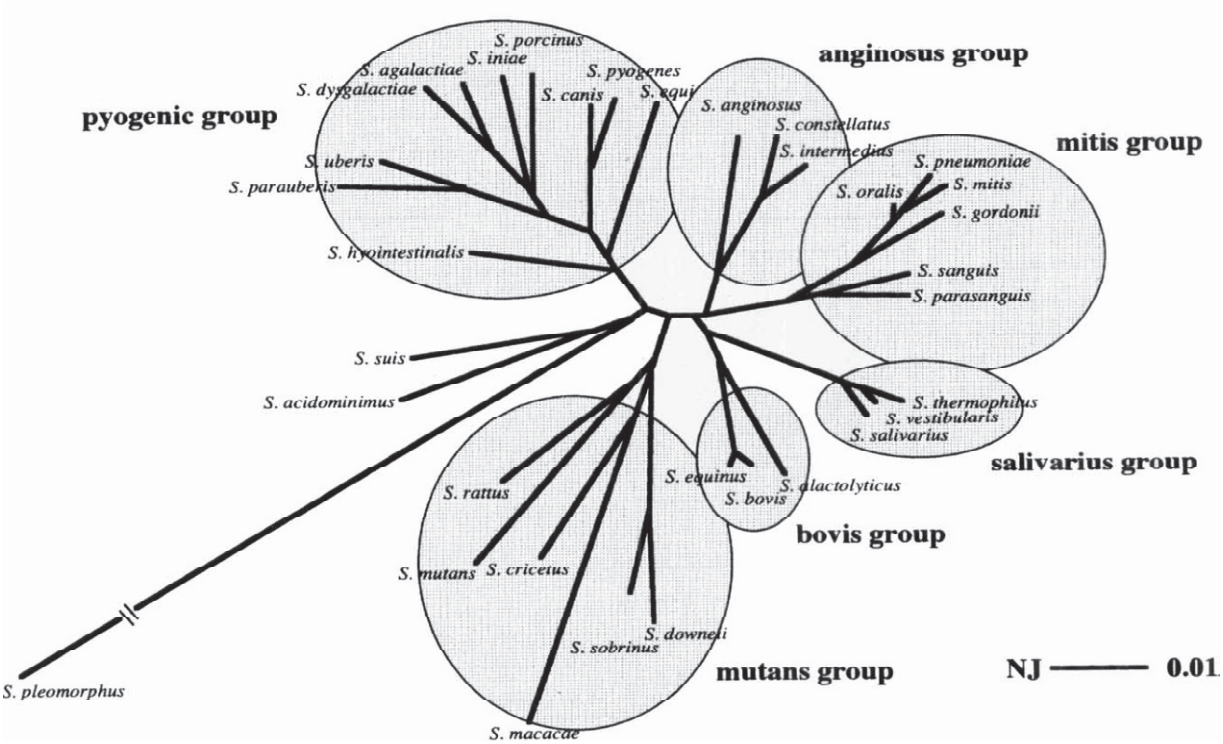

Figure 1: Phylogenetic relationships of 34 *Streptococcus* species calculated by the neighbour-joining method. Reprinted from Kawamura *et al.* (1995) with permission from Copyright Clearance Center.

1.1.2 General properties of streptococci

As mentioned above, the genus *Streptococcus* consists of Gram-positive bacteria with low genomic G+C contents (only 36-46%). Streptococcal cells are of spherical or ovoid shape, arranged in pairs (diplococci) or in chains of varying length. Streptococci are non-motile, non-spore forming, catalase negative and facultative anaerobe organisms (Hardie *et al.*, 1997). Members of this genus have a fermentative metabolism and complex nutritional needs that include vitamins, salts, amino acids, peptides, purines and pyrimidines. They generate ATP, reducing power and precursors for the synthesis of cellular material through the fermentation of carbohydrates. A characteristic trait of streptococci is actually their ability to ferment a wide range of sugars. The end product of this fermentation process in streptococci is mainly lactate, which is transported out of the bacterial cell as a waste product (Kilian, 1998).

1.2 *Streptococcus thermophilus*

S. thermophilus was first described by Orla-Jensen in 1919 (Sherman, 1937). This species is an essential thermophilic starter organism that is extensively used by the dairy industry worldwide. It has, as the only species of its genus, been classified as a GRAS (Generally Recognized As Safe) organism. This means that it is approved for food production and human consumption in the EU and USA. *S. thermophilus* is used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* or *L. delbrueckii* subsp. *helveticus* in the production of yoghurt, hard-cooked cheeses (e.g. Parmesan-types, Gruyère and Emmental), Cheddar and Mozzarella (Delorme, 2008). Some years ago it was estimated that these products together have an annual marked value of 40 billion U.S. dollars. This makes *S. thermophilus* the second most important dairy starter organism after *Lactococcus lactis* (Bolotin *et al.*, 2004; Hols *et al.*, 2005).

Only three different sugars are fermented by all strains of *S. thermophilus*, namely lactose, sucrose and glucose. In addition, some strains are able to grow on galactose and fructose. In contrast to most streptococci, *S. thermophilus* prefers lactose over glucose, demonstrating that it is highly adapted to growth in milk (Hols *et al.*, 2005). While catabolism of lactose is used for the generation of ATP, reducing power and precursor metabolites, catabolism of the major protein in milk (casein) provides precursors for the synthesis of

essential amino acids, vitamins and nucleotides (McSweeney *et al.*, 2000). The use of *S. thermophilus* in milk fermentation is based on its ability to quickly convert lactose into lactate at relatively high temperatures (40°C-45°C) (Sherman *et al.*, 1931). This leads to rapid reduction in pH that in combination with the high temperature inhibits growth of pathogens and spoilage organisms. *S. thermophilus* also contributes to the aroma and texture of dairy product (Leroy *et al.*, 2004). The accumulation of lactic acid acidifies the food, resulting in a tangy lactic acid taste. In addition, small amounts of aroma compounds such as formate, acetate, acetoin, acetaldehyde and diacetyl are produced during fermentation (Leroy *et al.*, 2004). In the ripening of cheeses, degradation of caseins to peptides and free amino acids adds to their background flavours, but even more important are a range of poorly understood catabolic reactions that convert free amino acids to volatile compounds essential for flavour (McSweeney *et al.*, 2000). Many strains of *S. thermophilus* also synthesise exopolysaccharides that confer improved rheological properties and desirable texture to fermented milk products (Garault *et al.*, 2000; Pastink *et al.*, 2009).

1.2.1 The genome of *Streptococcus thermophilus*

The genome of *S. thermophilus* was first sequenced by Bolotin *et al.* in 2004. At present the complete genome of four *S. thermophilus* strains (LMG 18311, CRNZ 1066, LMD-9 and JIM8232) are available at the National Center for Biotechnology Information (NCBI). This has provided new important insight into the genetics, metabolism and evolution of *S. thermophilus*.

S. thermophilus strains contain a circular chromosome of ~1.8 Mb with approximately 1900 protein-coding sequences. About 1500 of these 1900 coding sequences are orthologs to genes found in other streptococci. Interestingly, about 10% of the *S. thermophilus* genes are pseudogenes, which have lost their function due to frameshifts, nonsense mutations, deletions or truncations (Bolotin *et al.*, 2004). In comparison only 1-5% of the genes in other streptococcal species are pseudogenes (Bolotin *et al.*, 2004). The cellular functions most heavily affected by gene inactivation are those involved in energy metabolism. Approximately 30% of the genes involved in carbohydrate degradation, uptake and fermentation are truncated. Interestingly, half of the genes dedicated to sugar uptake are pseudogenes in *S. thermophilus*. Considering that the sugar available in milk is predominantly lactose, the loss of genes encoding redundant sugar transporters reflects adaptation of *S.*

thermophilus to a life in milk (Schroeter *et al.*, 2009). This is particularly well illustrated by the fact that *S. thermophilus* has acquired, presumably by lateral gene transfer, a lactose transporter (LacS) that is missing in other streptococci. LacS can function both as a lactose-H^+ symporter, driven by the proton motive force, and as a lactose/galactose antiporter. After lactose is transported into the cytoplasm it is cleaved into glucose and galactose by the cytoplasmic β-galactosidase. Since most strains of *S. thermophilus* cannot metabolize the galactose moiety of lactose, it is instead used to drive the uptake of lactose by LacS operating in the antiporter mode (Poolman, 2002; Hols *et al.*, 2005). Although current evidence suggest that *S. thermophilus* has primarily evolved though the loss-of-function mutations, footprints of many horizontal gene transfer (HGT) events show that HGT has played a decisive role in the shaping of the *S. thermophilus* genome. The occurrence of numerous small genomic islands, which for instance contain bacteriocin- and exopolysaccharide biosynthesis genes, are examples of this (Hols *et al.*, 2005). Particularly interesting is a 17 kb DNA fragment with 90% identity to a corresponding region in the genome of *S. thermophilus*' partner in yoghurt fermentation, *L. delbrueckii* subsp. *bulgaricus*. The acquisition of this fragment from *L. delbrueckii* subsp. *bulgaricus* has enabled *S. thermophilus* to synthesize the essential amino acid methionine, a rare amino acid in milk (Bolotin *et al.*, 2004).

Many genes that contribute to virulence in pathogenic streptococci are absent or only present as non-functional pseudogenes in *S. thermophilus.* Antibiotic resistance genes, for example, have not been detected in the genome of *S. thermophilus.* Neither have virulence-associated surface-exposed proteins that are known to enable pathogenic streptococci to adhere to mucosal surfaces and escape host defences. *S. thermophilus* has, for instance, only 4 surface-exposed proteins compared to 28 in *S. pneumoniae* (Zhang *et al.*, 1999). The ability to ferment a wide range of sugars has also been reported to be important for full virulence in *S. pneumoniae* and *Streptococcus pyogenes* (Ferretti, 2001; Tettelin *et al.*, 2001) presumably because it increases the ability of these pathogens to survive during infection. Consequently, it is reasonable to assume that the very limited sugar-metabolizing capacity of *S. thermophilus* has strongly reduced its pathogenic potential (Ferretti, 2001; Tettelin *et al.*, 2001; Ajdić *et al.*, 2002; Bolotin *et al.*, 2004). In sum, analyses of the genome sequence of *S. thermophilus* clearly shows that it evolved from an ancestor that possessed a number of virulence genes, and that these genes have been gradually lost during adaptation to the dairy niche.

1.3 Natural genetic transformation

Genomes are shaped by evolutionary processes such as mutations, gene duplication, gene loss and HGT. Comparative analyses of bacterial genomes has shown that HGT is a major driving force in the evolution of these organisms. Natural genetic transformation is one of three known mechanisms for HGT in bacteria (Lorenz *et al.*, 1994). The other two are transduction and conjugation. Bacteria that are competent for natural transformation have the ability to take up DNA from the environment and integrate it into their genomes by homologous recombination (Seifert *et al.*, 1988; Spratt, 1988; Gibbs *et al.*, 1989; Hoelzer *et al.*, 1991; Mongold, 1992; Davies, 1994; Spratt, 1994; Stevens *et al.*, 2011). In contrast to transduction and conjugation, natural genetic transformation is only initiated and dependent on the recipient cell. Natural transformation is part of the competent bacterium's normal physiology, and does not depend on foreign elements such as plasmids, transposons or bacteriophages. So far, more than sixty bacterial species from six different phyla (Deinococcus-Thermus, Cyanobacteria, Chlorobi, Proteobacteria, Firmicutes and Actinobacteria) have been demonstrated to be naturally transformable (Johnsborg *et al.*, 2007). However, since the core competence genes essential for DNA uptake and integration are present in the genome of most members of the Firmicutes and a number of bacteria belonging to other phyla, natural transformation is probably much more widespread than reported in the literature. Interestingly, no confirmed examples of Archaea that are competent for natural transformation have been reported. The reason for this might be that relatively few studies addressing this question have been conducted. Alternatively, the lack of naturally transformable Archaea may also indicate that this HGT mechanism is specific for the bacterial domain of life.

1.3.1 Natural genetic transformation in *Streptococcus pneumoniae*

Most of our current understanding of natural transformation comes from research carried out on the Gram-negative bacteria *Neisseria gonorrhoeae* and *Haemophilus influenza*, and on the Gram-positive bacteria *Bacillus subtilis* and *S. pneumoniae* (Solomon *et al.*, 1996). Natural transformation has been studied for more than eighty years in *S. pneumoniae*, and the pneumococcus has therefore become a paradigm organism for this phenomenon in Gram-positive bacteria. For this reason, the pioneering studies on natural transformation in *S. thermophilus* were based on what was known about the pneumococcal system. Consequently,

in order to better understand the rationale behind the research performed in this thesis, it is necessary to have background knowledge of natural transformation in *S. pneumoniae.*

S. pneumoniae holds a unique place in the history of molecular biology and genetics. In 1928, Frederick Griffith discovered "the transforming principle", while working with pneumococci. He found that, by a mechanism unknown to him, colony phenotypes could be transferred from dead to live pneumococci (Griffith, 1928). Then, in 1944, Oswald Avery and co-workers reported their ground-breaking finding that the transforming agent was, in fact, DNA. This was the first proof that DNA is the hereditary material of living organisms (Avery *et al.*, 1944). In the 1960s, it was reported that competence development in *S. pneumoniae* is a coordinated event taking place at a particular cell-density during exponential growth (Tomasz, 1965). Induction of the competence phenotype was demonstrated to be strictly regulated and to rely on a secreted proteinaceous compound. However, the exact nature of this compound remained elusive until the mid 1990s when Håvarstein *et al.* (1995a) finally reported the molecule to be a 17 amino acid peptide termed the competence stimulating peptide (CSP) (Håvarstein *et al.*, 1995a).

When grown under the appropriate conditions naturally transformable streptococci express a quorum-sensing system consisting of CSP, its dedicated secretion apparatus ComAB (Hui *et al.*, 1991), and a two-component regulatory system consisting of the histidine kinase ComD and its cognate response regulator ComE (Pestova *et al.*, 1996). CSP is encoded by the *comC* gene, which is cotranscribed with the *comDE* genes. CSP is synthesized as a precursor peptide containing a double-glycine type leader at its N-terminal end (Håvarstein *et al.*, 1995a; Håvarstein *et al.*, 1995b). The leader is cleaved off concomitant with export by a proteolytic domain located at the N-terminus of ComA. The external concentration of CSP is monitored by its membrane embedded receptor ComD (Håvarstein *et al.*, 1996), and upon reaching the critical level competence will be induced. By analogy to other two-component regulatory systems it is assumed that the binding of CSP to its receptor elicits autophosphorylation of ComD at a conserved histidine residue, and subsequent transfer of this phosphoryl group to an aspartate residue in the receiver domain of ComE. In its phosphorylated state ComE activates transcription of the alternative sigma factor ComX (Lee *et al.*, 1999; Claverys *et al.*, 2002) and about 20 additional early competence genes. Phosphorylated ComE binds as a homo-dimer to two tandem repeats (two 9 bp imperfect direct repeats separated by a 12 bp region) found in the promoter region of the early competence genes, including the *comAB* and *comCDE* operons (Ween *et al.*, 1999). This

"self-stimulation" mechanism, called an auto-induction loop, amplifies the response to CSP and eventually leads to expression of the alternative sigma factor ComX. ComX will in turn induce the transcription of the late competence genes (Lee *et al.*, 1999) (Fig. 2). It has not yet been experimentally proven that the *comX* promoter has a binding site for ComE, but a putative low-affinity direct repeat motif that is thought to bind phosphorylated ComE when the level inside the cell is sufficiently high, has been identified (Håvarstein, 2003).

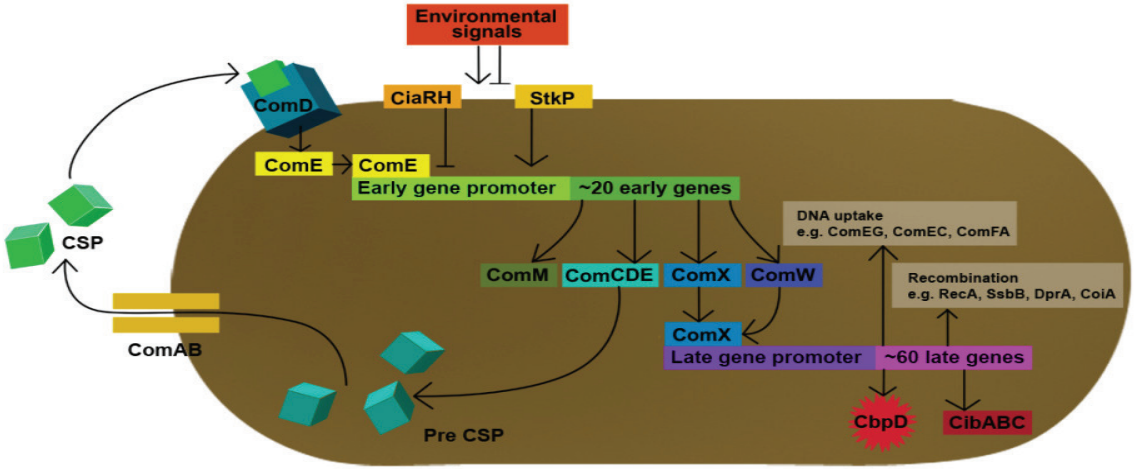

Figure 2: Model depicting competence regulation in *S. pneumoniae*. Expression of the *comCDE* genes depends on the growth conditions. Known regulators that monitor such conditions and control the basal transcription level of the *comCDE* operon are the serine/threonine protein kinase StkP and the CiaRH two-component system. Under conditions that are permissive for competence development, the ComAB and ComCDE proteins are expressed at low levels. ComC (pre-CSP), the precursor of the CSP pheromone, is processed and secreted by the dedicated ComAB transporter, resulting in a gradual extracellular accumulation of mature CSP. When the CSP concentration has reached a critical level, it activates phosphorylated ComE transcription of the early competence genes. One of the early genes, the alternative sigma factor ComX, subsequently activates transcription of the late competence genes. The late genes are often called the core competence genes, as their gene products are essential for DNA uptake and recombination. The key proteins of the fratricide mechanism are the immunity protein ComM and the murein hydrolase CbpD. Reprinted from Johnsborg *et al.* (2009) with permission from Wiley-Blackwell.

Alternative sigma factors are known to regulate transcription in bacteria by recruiting the RNA polymerase core enzyme to other promoters than the housekeeping promoters recognized by σ^A (Sharma *et al.*, 2010; Österberg *et al.*, 2011). Consequently, expression of an alternative sigma factor will turn on the transcription of a set of genes, often called a regulon, which are normally not expressed. In this way a bacterium can respond to stress,

changes in growth conditions, external stimuli etc. by synthesizing the appropriate gene products (Peterson *et al.*, 2004). In *S. pneumoniae* ComX binds the conserved consensus sequence, TACGAATA, also called the com-box or the cin-box, which are found in the promoter regions of most late competence genes (Campbell *et al.*, 1998; Peterson *et al.*, 2000). About 80 late competence genes have been identified so far (Peterson *et al.*, 2004). However, only 7 early and 14 late gene products are required for transformation under laboratory conditions (Dagkessamanskaia, 2004; Peterson *et al.*, 2004). The reason why expression of the remaining early and late genes is controlled by CSP is not known. Examples of late competence genes essential for transformation include genes encoding the competence pseudopilus required for DNA uptake (ComGA-D), the dsDNA receptor (ComEA), the ssDNA import channel (ComEC), an ATP-binding protein presumably providing energy for DNA uptake (ComFA), gene products involved in protection of internalized ssDNA (DprA and SsbB) and integration of this ssDNA into recipients genome through homologous recombination (RecA) (Claverys *et al.*, 2009).

1.4 Fratricide – a competence induced lysis mechanism

In 2002, it was discovered that chromosomal DNA is released into the growth medium upon competence induction in a culture of *S. pneumoniae* cells (Steinmoen *et al.*, 2002). Soon after, experiments with co-cultures of competence proficient and competence deficient (Δ*comE*) cells expressing the β-galactosidase reporter revealed that non-competent cells are actually lysed by their competent sister cells (Steinmoen *et al.*, 2003). In the years that followed, the Håvarstein group and others were able to show that a few of the CSP-responsive genes that are dispensable for transformation are part of a lysis mechanism called fratricide. Fratricide enables competent pneumococci to kill and lyse non-competent sister cells present in the same environment (Guiral *et al.*, 2005; Claverys *et al.*, 2007a; Claverys *et al.*, 2007b; Eldholm *et al.*, 2009; Johnsborg *et al.*, 2009). In *S. pneumoniae*, and its close relatives *Streptococcus mitis* and *Streptococcus oralis*, the key component of the fratricide mechanism is the secreted murein hydrolase CbpD. CbpD is encoded by a late competence gene, and is only expressed by competent cells (Kausmally *et al.*, 2005). To protect themselves against CbpD, competent pneumococci express an immunity protein, ComM, which is encoded by an early competence gene (Håvarstein *et al.*, 2006; Johnsborg *et al.*, 2008) (Fig. 3).

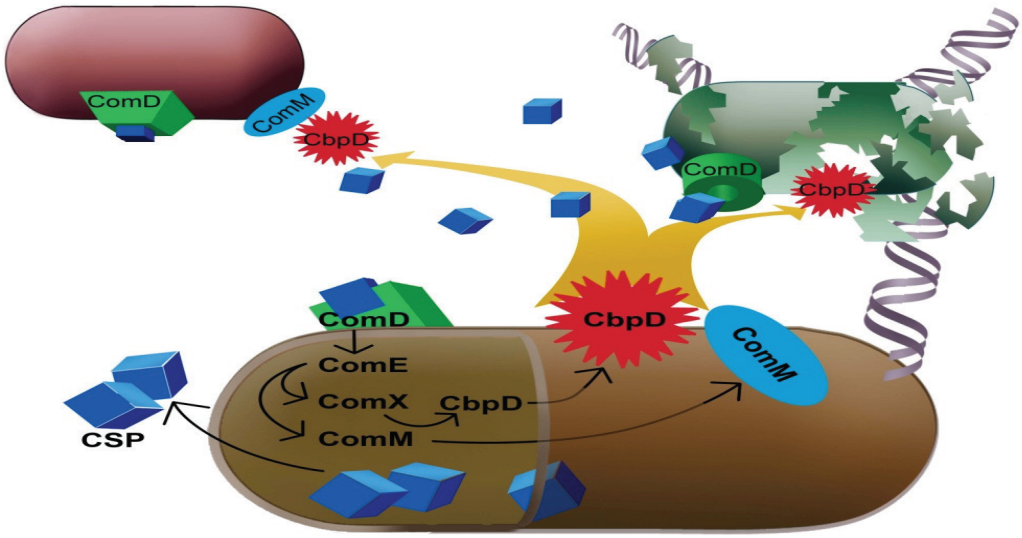

Figure 3: Model depicting how the fratricide mechanism is believed to function in *S. pneumoniae* and its close relatives *S. mitis* and *S. oralis*. When developing the competent state these streptococci (represented by the brown cell in front), attack and lyse their non-competent sister cells. Different strains and species of *S. pneumoniae, S. mitis* and *S. oralis* produce a number of different CSP pheromones. Each CSP type constitutes a separate pherogroup. Non-competent target streptococci (represented by the green cell) are sensitive because they belong to a different pherogroup, i.e. do not sense the CSP pheromone produced by the attacker cells, and consequently do not express the immunity protein ComM. The DNA released by the lysed cells is taken up by the competent attackers. In some cases streptococci (represented by the red cell) are able to sense non-cognate CSP-pheromones, i.e. CSPs produced by bacteria belonging to other pherogroups. These bacteria will detect the oncoming attack and be able to block the lytic effect of CbpD by expressing the ComM immunity protein. CSP, competence stimulating peptide; ComD, CSP histidine kinase receptor; ComE, the cognate response regulator of ComD; ComX alternative sigma factor controlling expression of the late genes; CbpD, murein hydrolase; ComM, immunity protein providing protection against CbpD. Reprinted from Johnsborg *et al.* (2008) with permission from Wiley-Blackwell.

CbpD is a modular murein hydrolase consisting of an N-terminal cysteine, histidine-dependent aminohydrolase / peptidase (CHAP) domain followed by two Src homology 3b (SH3b) domains and a C-terminal choline-binding domain (CBD) consisting of four choline-binding repeat units (Eldholm *et al.*, 2010). The exact chemical bond cleaved by the CHAP domain has not been identified, but CHAP is highly homologous to domains found in a number of phage lysins and bacterial murein hydrolases whose characterized members either act as endopeptidases that cleave within murein stem peptides, or as amidases that cleave the N-acetylmuramyl-L-Ala bond (Bateman *et al.*, 2003). Proteins harbouring CBDs have been shown to bind non-covalently to choline moieties decorating the wall- and lipoteichoic acids of *S. pneumoniae* and closely related streptococcal species (Sánches-Puelles *et al.*, 1986).

1.4.1 Impact of fratricide and lateral gene transfer

As shown in Fig. 3, expression of CbpD and ComM is co-regulated with natural transformation. Consequently, it has been proposed that the fratricide mechanism evolved to facilitate acquisition of homologous donor DNA from other pneumococcal strains and related streptococcal species (Claverys *et al.*, 2007a; Claverys *et al.*, 2007b; Johnsborg *et al.*, 2007). In a recent paper Johnsborg *et al.* (2008) provide strong evidence that this is indeed the case. They showed that transfer of an antibiotic resistance gene from non-competent target cells to competent attacker cells *in vitro* was a thousand-fold more efficient with wild type attacker cells than CbpD-deficient attacker cells. Furthermore, they demonstrated that the fratricide mechanism has a strong positive impact on the efficiency of gene transfer from the commensals *S. mitis* and *S. oralis* to *S. pneumoniae* (Johnsborg *et al.*, 2008). The fratricide mechanism depends on mixed populations of competent and non-competent bacteria in order to function. How do such populations arise in the natural habitat of pneumococci and their commensal relatives? Studies aimed at determining the number of CSP types produced by streptococcal strains and species have revealed that a large variety of CSP pheromones exists in nature. Due to this CSP diversity, mixed populations of competent and non-competent cells will presumably arise naturally in the multispecies biofilms where these streptococci live (Johnsborg *et al.*, 2008).

Pneumococcal CbpD will only be active against streptococci possessing choline-decorated teichoic acids in their cell walls. The reason for this is that CbpD needs both its SH3b- and CBD domains in order to bind to target cells (Eldholm *et al.*, 2010). Choline-decorated teichoic acid is a very unusual kind of teichoic acid that is only found in *S. pneumoniae* and a few closely related species; namely *Streptococcus pseudopneumoniae*, *S. mitis, S. oralis, Streptococcus peroris* and *Streptococcus infantis* (Kilian *et al.*, 2008). This implies that the fratricide mechanism has a limited target range. Furthermore, this makes biological sense, as it implies that the fratricide mechanism helps competent pneumococci and the above mentioned species to acquire relatively homologous DNA from related bacteria. The importance of discriminating between homologous and foreign DNA is illustrated by the fact that naturally transformable members of the Gram-negative families *Neisseriaceae* and *Pasturellaceae* have evolved a completely unrelated mechanism that serves the same purpose (Treangen *et al.*, 2008). These bacteria only take up DNA that contains 10-12 bp sequence motifs termed DNA uptake sequences (DUS). The role of the DUS sequences, which are evenly spread across the entire genomes of these bacteria, is to ensure that homologous rather

than potentially harmful foreign DNA is taken up by members of the *Neisseriaceae* and *Pasturellaceae*. In contrast, competent streptococci will take up any naked extracellular DNA regardless of source. For this reason, it is hypothesized that streptococci have evolved the fratricide mechanism to increase the chances of capturing homologous DNA during competence. Why is it so important for competent streptococci to acquire homologous DNA? Traditionally it has been assumed that the purpose of natural genetic transformation is to use genetic material from external sources to repair damaged genes, generate genetic diversity and/or acquire novel traits. In most cases, DNA from unrelated bacteria will not serve any of these purposes. The exception is that DNA acquired from an unrelated bacterium occasionally, but very seldom, contains novel genetic information that increases the recipients' competitiveness and chance of survival. However, in most cases foreign DNA will not be integrated into the recipients' genome, and if it does, it will in most cases be harmful to the host. In a very recent report Stevens *et al.* (2011) provide strong evidence that DNA damage that results in increased error rates during ribosomal decoding induces spontaneous competence development in *S. pneumoniae*. This finding suggests that DNA repair is the major biological function of natural transformation in streptococci (Stevens *et al.*, 2011). Damaged DNA can only be repaired with the corresponding undamaged DNA sequence from a closely related bacterium. Therefore, it is reasonable to assume that it is important for naturally transformable bacteria to possess a mechanism that enables them to predominantly take up homologous DNA.

1.5 Clp proteolytic complexes are involved in competence regulation in *Streptococcus pneumoniae*

Clp complexes are energy-dependent proteolytic machines that play an extremely important role in regulating gene expression and removing damaged proteins from cells. In the low G+C Gram-positive bacteria Clp proteins are central in coordinating stress responses and various development decisions (Frees *et al.*, 2007). Clp complexes consist of two different functional elements that are arranged as a stack of rings. One element, termed ClpP, is a serine protease that consists of two stacked heptameric rings, which together enclose a chamber where proteolysis of target proteins take place. The other element is the hexameric ATPases, which independently of ClpP harbour chaperone activity (Fig. 4a). When bound to

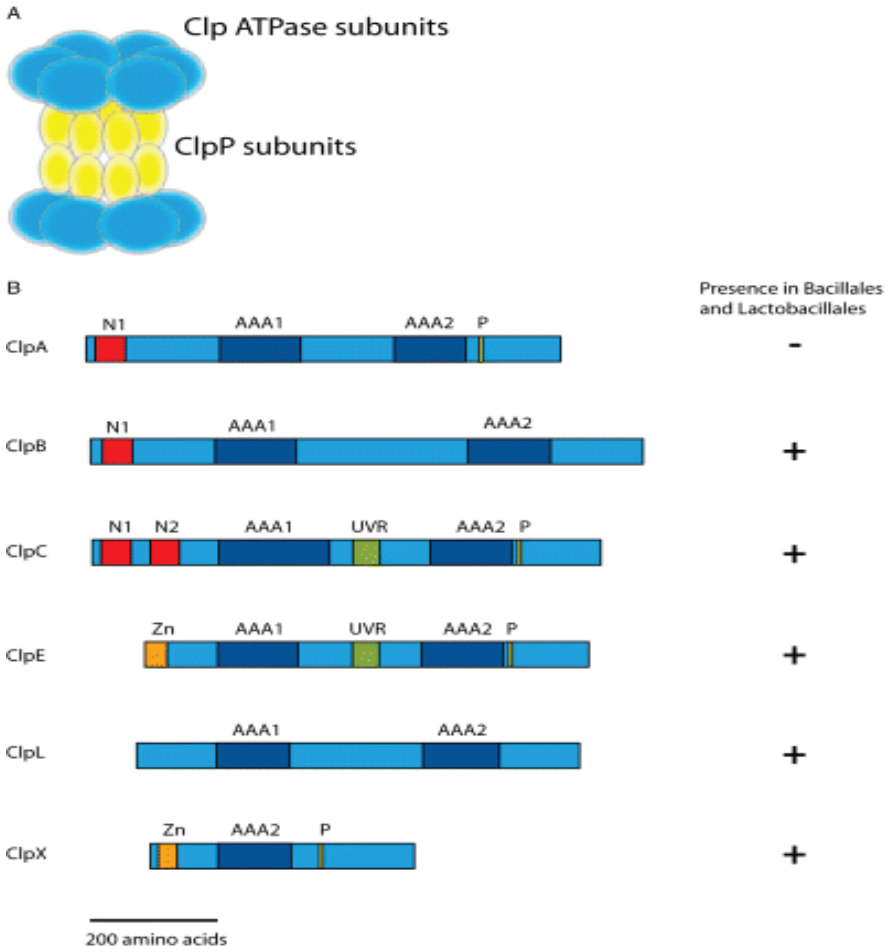

Figure 4: (A) Model depicting the structural organization of the ClpP proteolytic complex, (B) Domain organization of the Clp ATPase subfamilies present in the *Bacillales* and *Lactobacillales*. The Clp ATPases have either one or two nucleotide binding domains (AAA-1 and/or AAA-2). The length of the spacing between these domains, as well as the presence of specific signature sequences, forms the basis for the subfamily classification (Schirmer *et al.*, 1996; Ingmer *et al.*, 1999; Porankiewicz *et al.*, 1999). Functional domains include the P domain required for binding to ClpP (Kim *et al.*, 2001), the Zn binding domain involved in dimerization (Wojtyra *et al.*, 2003) and the N1 and N2 domains proposed to be involved in the protein binding (Barnett *et al.*, 2005). A domain (UVR) resembling the interaction domain between the nucleotide excision repair proteins, UvrB and UvrC, has also been identified in several Clp ATPases (Ingmer *et al.*, 1999). Figure reprinted from Frees *et al.* (2007) with permission from Wiley-Blackwell.

ClpP these ATPases determine target specificity, a process often modulated by an additional adaptor protein, such as MecA or ClpS (Frees *et al.*, 2007; Kress *et al.*, 2009). In addition to ClpP, *S. pneumoniae* encodes four different Clp ATPases, namely ClpC, ClpE, ClpL and ClpX (Robertson *et al.*, 2003). The same *clp* genes are present in the genome of *S. thermophilus* (Bolotin *et al.*, 2004).

In *S. pneumoniae*, several reports describe that ClpP contributes to virulence, thermotolerance and oxidative stress resistance (Robertson *et al.*, 2002; Robertson *et al.*,

2003; Kwon *et al.*, 2004; Ibrahim *et al.*, 2005). Compared to *S. pneumoniae*, the role of the Clp proteins have been less investigated in *S. thermophilus*. However, ClpL has been shown to be essential for growth at 50°C in this species (Varcamonti *et al.*, 2006; Li *et al.*, 2011). Interestingly, it has been reported that the Clp system plays a role in the regulation of competence development in *S. pneumoniae*. When grown under conditions inappropriate for competence development, transcription of the *comCDE* genes is repressed. Chastanet *et al.* (2001) discovered that this repression is at least partly abolished in a *clpP* mutant (Chastanet *et al.*, 2001). Later, Morrison and co-workers found that the efficiency by which ComX induces transcription of the late competence genes is modulated by ComW, a protein encoded by an early competence gene (Luo *et al.*, 2004; Sung *et al.*, 2005). Experimental evidence suggests that ComW protects ComX against ClpP mediated proteolysis. In addition, it has been reported that ComW stimulates the activity of ComX by an unknown mechanism (Sung *et al.*, 2005).

1.6 Natural genetic transformation in *Streptococcus thermophilus*

All attempts to make *S. thermophilus* competent for natural genetic transformation in the laboratory failed until recently. Thus, *S. thermophilus* has traditionally been considered to lack this property. However, when its complete genome sequence was published in 2004 (Bolotin *et al.*, 2004), it became possible to search for genes encoding proteins that are known to be essential for natural transformation in *S. pneumoniae*. Interestingly, BLASTP searches in the NCBI database revealed that *S. thermophilus* contains homologues of the core competence genes, i.e. the genes essential for DNA uptake and recombination. In addition, a gene encoding a homologue of ComX was detected (Hols *et al.*, 2005). However, the early competence genes of *S. pneumoniae*, including those encoding the ComABCDE signal transduction pathway, appeared to be missing. This finding indicated that *S. thermophilus* might be naturally transformable after all, provided that the indentified competence genes are still functional. It also indicated that the competence state in *S. thermophilus* is activated by a novel mechanism unrelated to the ComABCDE pathway.

A breakthrough in this area of research was reported by Blomqvist and co-workers, who were able to make *S. thermophilus* LMG 18311 competent for natural transformation by ectopic expression of ComX from a peptide pheromone-inducible bacteriocin promoter (Blomqvist *et al.*, 2006a; Blomqvist *et al.*, 2006b). This result demonstrated that the *comX*

gene and the core competence genes of *S. thermophilus* LMG 18311 are functional. The inducible expression system used by Blomqvist *et al.* was based on a quorum sensing system StbABCHR, which regulates bacteriocin production in *S. thermophilus*. The ABC-transporter StbA and its accessory protein StbB constitute a dedicated secretion apparatus that translocates the *Streptococcus thermophilus* pheromone (STP) (NH_2-SGWMDYINGFLKGF-GGQRTLPTKDYNIPQV-COOH) across the cytoplasmic membrane. STP is encoded by the *stbC* gene, and is synthesized as a precursor peptide with an N-terminal leader of the double-glycine type. The external concentration of STP is sensed by the transmembrane histidine kinase StbH, which upon binding of the pheromone transfers a phosphoryl group to the response regulator StbR. Phosphorylated StbR then activates transcription by binding to a direct-repeat motif found in the promoters of several different bacteriocin genes present in the genome of *S. thermophilus*. The *comX* gene was cloned into the shuttle plasmid pTRKH2 (O'Sullivan *et al.*, 1993) behind the promoter of the gene encoding the bacteriocin termed StbD. The resulting pXL plasmid was introduced into the *S. thermophilus* LMG 18311 strain by electroporation. Strains containing the pXL plasmid were induced to competence by addition of 250 ng/ml of STP in the early logarithmic phase of growth (Blomqvist *et al.*, 2006a; Blomqvist *et al.*, 2006b).

An important question left unanswered in the study of Blomqvist *et al.* (2006a) was whether *S. thermophilus* has lost the genes controlling ComX expression, or if this species possesses a competence induction pathway that is completely unrelated to the ComABCDE system used by *S. pneumoniae* and its relatives in the mitis phylogenetic group. It is known from the literature that an alternative signal transduction pathway used for cell-cell communication exists in Gram-positive bacteria (Monnet, 2003). This pathway is based on an oligopeptide permease, which transports the signalling peptide into the cytoplasm, and an intracellular receptor. The receptor functions as a transcriptional activator that becomes active when it forms a complex with the signalling peptide. When investigating whether the two Ami oligopeptide permeases (AmiA1 and AmiA3) produced by *S. thermophilus* strain LMD-9 play a role in competence regulation, Gardan *et al.* discovered that this strain develops the competent state spontaneously when cultivated in a chemically defined medium (CDM) (Gardan *et al.*, 2009). They were also able to show that deletion of both *ami* genes abolished competence development, indicating that a signalling peptide imported by the Ami system is involved in competence regulation in *S. thermophilus.* Their choice of the LMD-9 strain was

lucky, as no spontaneous competence development was observed when the CRNZ1066 and LMG18311 strains were cultivated in CDM.

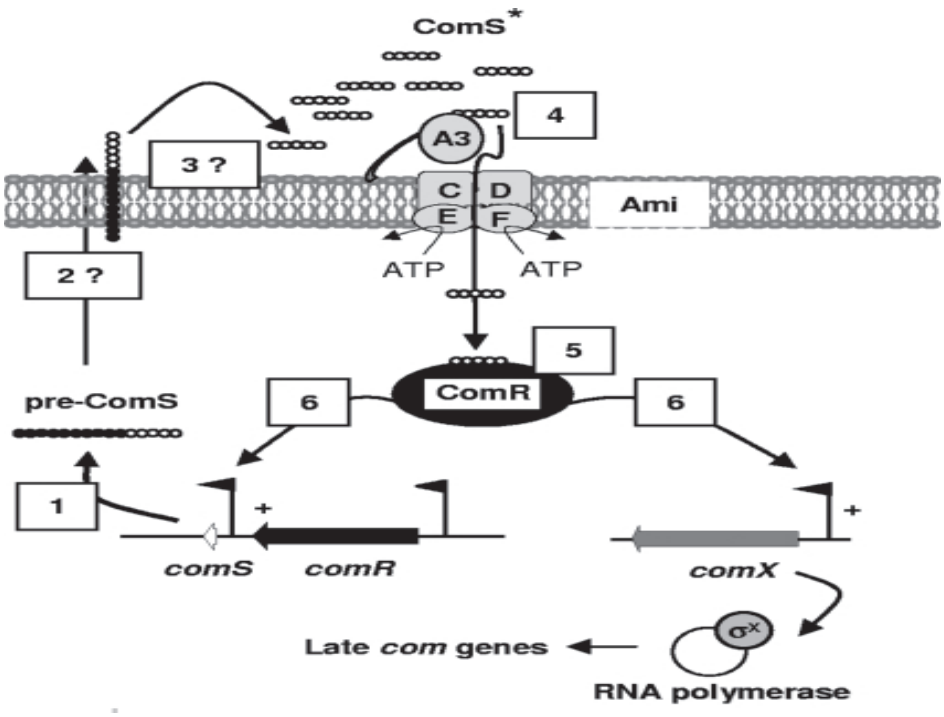

Figure 5: Schematic representation of the quorum-sensing-like competence-induction pathway of *S. thermophilus.* Under growth conditions that are permissible for spontaneous competence development the precursor of the ComS* signalling peptide (pre-ComS) is synthesized (1). The precursor is processed and exported to the extracellular medium by an unknown mechanism (2 and 3). Extracellular ComS* is recognized by the oligopeptide-binding protein AmiA3, and imported into the cytoplasm by the AmiCDEF oligopeptide transporter (4). Once inside the cytoplasm ComS* presumably binds to and activates the ComR transcriptional activator (5). In its active state ComR is believed to bind to the promoters of *comS* and *comX* to amplify the signal and induce expression of ComX (6). Eventually this leads to expression of the late competence genes and development of the competent state. Black flags represent promoters, while + represent activation by ComR. Reprinted from Fontaine et al. (2010) with permission from ASM.

The gene (*sph0316*) encoding the *S. thermophilus* competence pheromone was identified the year after by Fontaine and colleagues (Fontaine *et al.*, 2010). It turned out to encode a small hydrophobic protein consisting of only 24 amino acids. Chemical synthesis of different parts of this protein showed that a peptide consisting of the 8 C-terminal amino acids (NH_2-LPYFAGCL-COOH) has biological activity. This synthetic peptide was termed ComS*. Fontaine *et al.* identified the cytoplasmic receptor of ComS* as well. The receptor, ComR (ster_0316), which belongs to the Rgg-family of transcriptional regulators, is located immediately upstream of the unannotated *sph0316* gene (Fig.5) (Fontaine *et al.*, 2010).

How the 24 amino acid precursor ComS* is externalized and processed remains to be determined. Another key question that remains to be answered concerns the regulation of ComS* production. As shown by Gardan *et al.*, spontaneous competence development in strain LMD-9 depends on the growth conditions, strongly indicating that ComS* production is regulated by environmental signals. This discovery of the ComRS system in *S. thermophilus* prompted Mashburn-Warren *et al.* (2010) to look for similar systems in other streptococcal species possessing the core competence genes, but lacking a ComABCDE-type competence induction pathway. Using an *in silico* approach, they found that *comRS*-related genes are present in the genomes of several streptococci belonging to the pyogenic and bovis phylogenetic groups, suggesting that competence in these species is regulated by a ComRS-type system. Curiously, they found that *S. mutans* possesses a ComRS competence induction pathway in addition to the previously described ComABCDE pathway. Activation of both of these pathways induces the competent state in *S. mutans*. However, while ComRS is indispensable for competence, the reverse is not true. This result shows that ComR and not ComE is the proximal activator of ComX in *S. mutans* (Mashburn-Warren *et al*., 2010).

2. BACKGROUND and AIMS OF THE STUDY

Natural genetic transformation is one of three horizontal gene transfer mechanisms operating in bacteria. Over the years, an increasing number of streptococcal species have been found to possess this property, and it is now believed that all members of this genus can become competent for natural transformation under the right circumstances (Håvarstein, 2010; Mashburn-Warren *et al.*, 2010). For streptococci this mechanism represents a major evolutionary driving force that plays an important role in the shaping of their genomes and general lifestyles. To better understand streptococci, it is necessary to understand how they use natural transformation to survive in an adverse and ever-changing environment.

The general purpose of the current study was to gain new and deeper insight into the natural transformation mechanism of *S. thermophilus*. Two aspects of this mechanism were of particular interest. One of them concerned the regulation of ComX expression. *S. thermophilus* lacks the ComABCDE-type signal transduction pathway found in *S. pneumoniae* and related streptococci, and we therefore speculated that it uses an alternative way to activate transcription of the *comX* gene. Since the Clp system was known to influence competence development in *S. pneumoniae* we decided to investigate whether the same is true for *S. thermophilus*. The purpose of the study was to determine whether the Clp system is part of the regulatory pathway(s) controlling competence development in *S. thermophilus* (Paper I).

The other point of interest concerned the fratricide mechanism previously discovered in *S. pneumoniae*. *In silico* searches for *cbpD*-like genes in streptococcal genomes had shown that such genes are present in the genomes of *S. thermophilus* and a number of other streptococcal species. The widespread occurrence of these genes indicates that they play an important biological role associated with natural competence. Since *S. thermophilus* and *S. pneumoniae* have adapted to very different lifestyles, we reasoned that new insight into the fratricide mechanism could be gained by studying this phenomenon in *S. thermophilus* (Paper II).

During the course of the present study, major breakthroughs were made by the research groups of Véronique Monnet and Pascal Hols that led to the identification of the ComRS competence induction pathway of *S. thermophilus* (Gardan *et al.*, 2009; Fontaine *et*

al., 2010). As the only research group studying natural transformation in both *S. thermophilus* and *S. pneumoniae* we saw the possibility of exploiting the newly identified ComRS system for controlled gene expression and depletion in *S. pneumoniae*. *S. pneumoniae* lacks close homologues of the *comRS* gene products, and the use of this system for ectopic gene expression should therefore not interfere with the normal physiology of the pneumococcus. Development of a reliable and easy to use gene depletion system for the study of pneumococcal genes that are essential for viability would be a very valuable tool for future research (Paper III).

3. RESULTS and DISCUSSION

ClpC acts as a negative regulator of competence in *Streptococcus thermophilus*

The genomes of *S. thermophilus* and *S. pneumoniae* encode the same Clp proteins, namely ClpP, ClpC, ClpE, ClpL and ClpX. Similarly to what had previously been reported for *S. pneumoniae*, we found that a *S. thermophilus clpX* deletion mutant is not viable. Furthermore, deletion of the *clpP* gene of *S. thermophilus* gave rise to only a few transformants, all of which grew very poorly. As these *clpP* mutants obviously were under great stress and for this reason probably would accumulate suppressor mutations, we decided not to study them any further. Viable *clpP* mutants, however, were obtained with *S. pneumoniae* strains R6 and CP1250 (Chastanet *et al.*, 2001; Luo, 2003). Robertson *et al.* (2002), on the other hand, reported that a *clpP* mutant of *S. pneumoniae* D39 grew poorly (Robertson *et al.*, 2002). Judging from the studies carried out with *clpP* mutants of the R6 and CP1250 strains, ClpP acts as a negative regulator of competence in *S. pneumoniae.* As deletion of the *clpP* gene leads to increased accumulation of ComX in competent pneumococci, it seems likely that a negative effect of ClpP on spontaneous competence development is caused by ClpP-mediated degradation of ComX (Sung *et al.*, 2005). A similar result was obtained by Opdyke *et al.* (2003) who observed that deletion of the *clpP* gene in *S. pyogenes* caused increased accumulation of ComX compared to the wild-type strain (Opdyke *et al.*, 2003).

Regrettably, for the reason mentioned above, we were not able to study the effect of deleting *clpP* on competence development in *S. thermophilus.* Interestingly, however, deletion of *clpC* and *clpE* both had a strong positive impact on late gene expression in STP-induced and uninduced cells carrying the pXL plasmid (Paper I, Fig. 1a and b). The effect of knocking out *clpC* was more pronounced, and we therefore chose to focus our further studies on this Clp protein. In the *clpC* mutant, the positive effect, as measured by the activity of the luciferase reporter, was equally strong even in the absence of STP induction. In the absence of STP induction only relative small amounts of ComX will be produced due to the leakiness of the bacteriocin promoter (Blomqvist *et al.*, 2006b). The fact that the light produced by the luciferase reporter was equally strong in induced and non-induced cells must mean that enough ComX accumulated in non-induced cells to fully activate the *comEC* late gene promoter. There are two possible explanations for this result: Either more ComX is produced

in the Δ*clpC* background, or ComX is more stable in the absence of ClpC. Our data support the latter alternative. This is based on the result showing that transcription from the *comX* promoter is not upregulated in a *clpC* mutant (Paper I, Fig. 1d). In addition, our data showed that in contrast to the wild-type strain, a *clpC* mutant carrying the pEAP plasmid produced significant amounts of light (Paper I, Fig. 1c). The pEAP plasmid contains the *luc* gene under the control of the *comEC* late gene promoter. Deletion of the native *comX* gene in the Δ*clpC* strain carrying the pEAP plasmid cancelled out the light production, demonstrating that more ComX accumulates in *S. thermophilus* cells lacking ClpC (unpublished results). It follows from these results that ClpC in complex with the proteolytic subunit ClpP probably degrades ComX, and thereby acts as a negative regulator of competence in *S. thermophilus*.

Experiments carried out to compare the transformability of ClpC-proficient and ClpC-deficient *S. thermophilus* cultures showed that low level competence develops spontaneously in the latter. These transformation experiments also showed that spontaneous competence development in Δ*clpC* cultures was tenfold higher in the semisynthetic C medium than in THG broth. This in accordance with results of Gardan *et al.* (2009), which discovered that the use of a chemically defined medium induced competence in the *S. thermophilus* LMD-9 strain (Gardan *et al.*, 2009). The fact that the largest difference in transformability between ClpC-proficient and ClpC-deficient *S. thermophilus* cells was obtained in uninduced cultures, i.e. in cultures not treated with STP or ComS*, again demonstrated that the effect of ClpC is best seen when the level of ComX is low (Paper I, Table 2).

To verify that ClpC mediates degradation of ComX, the *comX* gene in the pXL plasmid was exchanged with a His-tagged version. Using this construct, the intracellular concentration of ComX could be monitored by Western analysis using the Pierce SuperSignal West Pico HisProbe kit. In accordance with our hypothesis, the level of ComX-His increased more rapidly in Δ*clpC* than in wild-type cells after induction of ComX expression by STP at $OD_{492} = 0.05$ (Paper I, Fig. 3). However, unexpectedly, when cultures reached $OD_{492} = 0.4$ the level of ComX was higher in the wild-type than in the *clpC* mutant cells. At present we are not able to explain this result, but we think that part of the explanation is that ClpC becomes saturated due to the fact that the STP-induced bacteriocin promoter driving ComX-His expression is very strong. This assessment is based on a comparison of the amount of light emitted when different promoters drive expression of the *luc* reporter gene (unpublished results). Saturation of ClpC explains why the level of ComX continues to increase in the wild-

type strain, but does not explain why it decreases at higher cell densities in the *clpC* mutant (Paper I, Fig. 3). Further research is needed to clarify these issues.

The serendipitous discovery of a competence-up mutant, T2, which turned out to be a mutant with a non-functional *clpC* gene, gave us the opportunity to further explore the contribution of ClpC with respect to competence regulation in *S. thermophilus.* To ensure that the $G_{1630} \rightarrow T_{1630}$ nonsense mutation that introduced a premature stop-codon in the *clpC* gene plays a part in the competence-up phenotype, we back-mutated the nonsense mutation to the wild-type sequence. Since the resulting T2-R strain as well as the T2 parental strain carry the pXL plasmid, their competence phenotypes could be compared by measuring *luc* activity in the presence and absence of the STP peptide. The result showed that uninduced culture of the T2-R strain produced much less light than the corresponding T2 culture (Paper I, Fig. 2a and d). Similarly, the transformation efficiency obtained with the T2-R strain was significantly reduced compared to the T2 strain (Paper I, Table 2).

Together our findings support the view that ClpC is able to suppress competence when the level of ComX in the cell is low. The biological function of ClpC with respect to competence regulation is therefore probably to prevent that ComX accumulates in *S. thermophilus* and induces competence at the wrong time and place, i.e. under conditions where the exogenous and/or endogenous signals promoting competence development are weak.

Deletion of the murein hydrolase CbpD reduced transformation efficiency in *Streptococcus thermophilus*

BLASTP searches revealed that the genome of *S. thermophilus* encodes a CbpD-like protein (Claverys *et al.*, 2007b). This protein, which we have termed CbpD-St to distinguish it from the pneumococcal counterpart CbpD-Sp, possesses a CHAP domain in its N-terminus that is highly homologous to the corresponding N-terminal domain found in CbpD-Sp. In addition, the gene encoding CbpD-St has a com-box in its promoter region, indicating that it belongs to the late competence genes. However, as shown in Fig. 6, the C-terminal domains of CbpD-St and CbpD-Sp are completely unrelated. As the C-terminal domain of CbpD-Sp has been shown to anchor the neighbouring muralytic CHAP domain to the cell surface of

target cells (Eldholm *et al.*, 2010), we speculated that the C-terminal domain of CbpD-St serves the same function.

In pneumococci, CbpD-Sp, which is produced exclusively by competent cells, is the key component of the fratricide mechanism. Competent pneumococci commit fratricide when they grow in mixed cultures with non-competent pneumococci or related species such as *S. mitis* and *S. oralis* (Johnsborg *et al.*, 2008). CbpD-Sp lyse non-competent cells by attacking and breaking down their cell walls, whereas competent cells are protected against CbpD-Sp by the immunity protein ComM. Considering that *S. thermophilus*, in contrast to *S. pneumoniae*, is a domesticated species that presumably has lived in a dairy environment for thousands of years, we wanted to compare the properties of CbpD-St and CbpD-Sp to see whether they serve similar functions or have evolved to play different roles in their respective species.

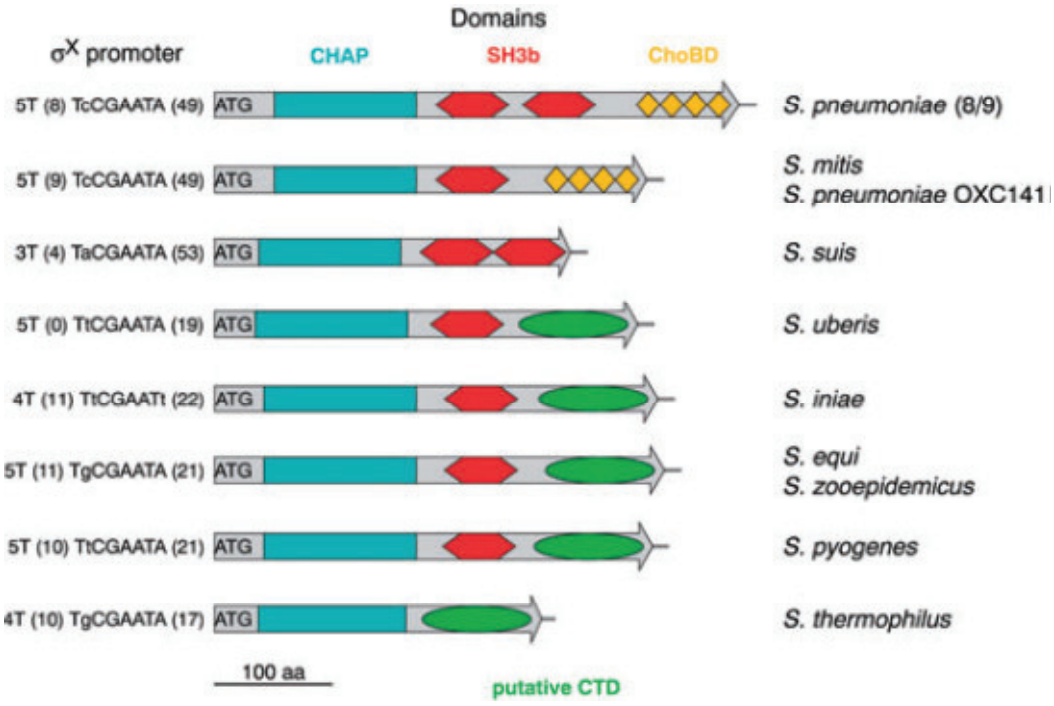

Figure 6: Schematic representation of CbpD-like proteins from different streptococcal species. The proteins were identified by BLASP searches using the pneumococcal CHAP domain as a query sequence. Blue, CHAP domains; red, SH3 domains; yellow, choline-binding domains and green, domains with an unknown function. Reprinted from Claverys *et al.* (2007b) with permission from Wiley-Blackwell.

It has been reported previously that deletion of *cbpD-Sp* has no effect on the transformability of *S. pneumoniae* strain R6 (Johnsborg *et al.*, 2008), strongly indicating that this murein hydrolase is not directly involved in DNA binding or uptake. The same result was

obtained for LytF, a competence induced murein hydrolase produced by *Streptococcus gordonii* that is essentially unrelated to CbpD-Sp. Similar to CbpD-Sp, LytF contributes strongly to increase the efficiency of gene transfer between donor and recipient cells *in vitro* (unpublished results, Berg, K.H., Ohnstad, H. and Håvarstein, L.S.). However, in contrast to the pneumococcal fratricide system, no detectable immunity against LytF was displayed by competent *S. gordonii* cells. Presumably, *S. gordonii* and other streptococcal species possessing LytF-type fratricins protect themselves by a different kind of mechanism that is not directly linked to induction of the competent state. Perhaps a population of *S. gordonii* cells are naturally heterogeneous with respect to their susceptibility towards LytF. It is well known that cells growing under identical conditions might differentiate into distinct subpopulations, a phenomenon often called bistability (Dubnau *et al.*, 2006). If this population-heterogeneity hypothesis turns out to be correct, fratricide in *S. gordonii* appears altruistic in nature rather than predatory.

Since neither CbpD-Sp nor LytF affects the transformability of their respective hosts, it came as a big surprise when we discovered that a *S. thermophilus cbpD-St* mutant (ST0039-1) displayed about eighteen-fold lower transformation efficiency than the wild-type strain (Paper II, Table 3). Our immediate reaction was that the result could be due to a polar effect of the inserted kanamycin-resistance marker, or some other flaw in the experimental design. We therefore constructed a *cbpD-St* mutant (ST0039-2) with the kan^R gene oriented in the opposite direction. In this case the reduction of transformability was about seventeen-fold compared to the wild-type. To further convince ourselves that the reduced transformability obtained with ST0039-1 and ST0039-2 was caused by the lack of the CbpD-St protein, we attempted to complement the mutation by expressing CbpD-St ectopically in the strain termed ST0039-C. To make the ST0039-C strain, the *cbpD-St* gene and its promoter was cloned into the pTRKH2 plasmid and introduced into the ST0039-2 strain by electroporation. The natural transformability of the ST0039-C strain turned out to be about the same as the wild-type, demonstrating that ectopic expression of CbpD-St was able to complement the loss of the native *cbpD-St* gene (Paper II, Table 3).

As a final control we tested whether the nature of the transforming DNA might influence the results. The p0055 plasmid, used as donor DNA in the experiments discussed above, carries a kanamycin-resistance gene flanked by the immediate up- and downstream regions of the *stu0055* gene. Insertion of the kan^R gene into the genome of *S. thermophilus* by double-crossover recombination will result in deletion of the *stu0055* gene. This small gene

has no known function, and can be deleted without causing any apparent harm to *S. thermophilus.* When exchanging this donor DNA with genomic DNA isolated from a rifampicin resistant *S. thermophilus* strain, the overall transformation efficiency went down about 200-fold. The reason for this is that the genomic rif^R marker constitutes a much smaller part of the total DNA used for transformation than the plasmid-borne kan^R marker. However, even with the rif^R DNA, the transformability of the ST0039-2 strain was 6.5 times lower than the wild-type (Paper II, Table 3). In sum, these results clearly show that without a functional CbpD-St protein, the transformation efficiency of naturally competent *S. thermophilus* cells is reduced.

What could be the mechanism behind CbpD-St's stimulatory effect on transformation efficiency? To come up with a plausible hypothesis we needed to know more about the properties of CbpD-St. The first thing we wanted to find out was whether CbpD-St functions as a murein hydrolase. This was done by running total protein extracts from competent and non-competent wild-type and ST0039-2 (Δ*cbpD-St*) cells on a SDS-PAGE gel containing whole heat inactivated wild-type cells as substrate. After washing the zymogram in deionized water to remove SDS, it was incubated in refolding buffer. A clearing zone corresponding to the CbpD-St protein appeared in the lane loaded with extract from competent wild-type cells, but not in lanes loaded with other samples (Paper II, Fig. 1). This result shows that CbpD-St is a murein hydrolase, and that it is produced only by competent cells. Thus, in these respects it is a functional analogue of CbpD-Sp. To determine whether the C-terminal domain of CbpD-St anchors the protein to the surface of *S. thermophilus* cells, a fusion protein in which the CHAP domain of CbpD-St had been exchanged with GFP, was constructed. Subsequent analysis, by fluorescence microscopy, revealed that the fusion protein predominantly binds to the equatorial region of *S. thermophilus* cells (Paper II, Fig. 2). In contrast, a corresponding GFP-CbpD-Sp fusion protein constructed in the same way, bound to the cell equator as well as the septal and polar regions of pneumococcal cells (Eldholm *et al.*, 2010). This difference in binding specificity might reflect that CbpD-St and CbpD-Sp have different biological roles. However, most likely both act as lysins that initiate their lethal muralytic activity by binding to different cell surface structures in their respective species.

Having confirmed that CbpD-St is a murein hydrolase, we wanted to investigate whether it is able to cause cell lysis in a competent culture of *S. thermophilus* cells. To assess the level of cell lysis we measured the β-galactosidase activity in sterile-filtered growth medium and compared it to total β-galactosidase activity in the culture (extracellular +

intracellular activity). In *S. thermophilus* β-galactosidase is an intracellular enzyme, and it can therefore be used to detect cell lysis. The results showed that about 4% of the cells lysed in a competent culture, and that this lysis resulted from the activity of CbpD-St (Paper II, table 4). Interestingly, however, more than 30% of the competence induced cell population lysed when 1 mM dithiothreitol (DTT) was added to the culture (Paper II, Table 4 and Fig. 3). Addition of 400 U/ml of catalase also had a strong positive effect on the activity of CbpD-St, resulting in lysis of about 13% of the population. Together, these results show that CbpD-St is prone to oxidation. Most likely CbpD-St is inactivated by oxidation of the active site cysteine in its CHAP domain. The same phenomenon has been observed previously for CbpD-Sp (Eldholm *et al.*, 2009). Considering the high homology between the CHAP domains of CbpD-St and CbpD-Sp, this was to be expected. The stimulating effect of the added catalase shows that H_2O_2 produced by *S. thermophilus* cells rapidly inactivates extracellular CbpD-St that is secreted during competence. In *S. thermophilus* H_2O_2 production depends on Mn-superoxide dismutase (SodA), which converts superoxide anions (O_2^-) to O_2 and H_2O_2 (Hols *et al.*, 2005). Since synthesis of H_2O_2 depends on the presence of oxygen, its production will presumably cease under anaerobic growth conditions. Consequently, inhibition of CbpD-St by H_2O_2 would not represent a problem if *S. thermophilus* requires anaerobic growth conditions to undergo spontaneous competence development. So far, spontaneous competence development in *S. thermophilus* has only been observed by Gardan *et al.* (2009) with strain LMD-9. In this case, spontaneous competence development required a chemically defined medium, but not anaerobic growth conditions. To synthesize and export an enzyme that is almost immediately inactivated seems like a wasteful process that evolution should strongly select against. We therefore speculated that the observed inactivation of CbpD-St by H_2O_2 represents a protective mechanism that ensures that only a limited fraction of the cell population is lysed during competence.

What, then, is the biological function of CbpD-St? Our results suggest that there are two possibilities: CbpD-St either functions as a lytic fratricin directed against other bacteria, or as a facilitator of DNA uptake that increases the permeability of the *S. thermophilus* cell wall by making controlled cuts in the peptidoglycan layer. In our view, it is most likely that CbpD-St functions as a fratricin that lyses susceptible cells in order to release homologous transforming DNA that can be taken up by the surviving competent cells in the population. As mentioned in the introduction section, Stevens *et al.* (2011) have recently provided strong evidence that competence for natural transformation in *S. pneumoniae* is important for DNA

repair. Such repair should be equally important for domesticated species, such as *S. thermophilus*. Even though *S. thermophilus* cells grow in a nutrient-rich, protected and stable environment with relatively little competition from other bacteria, they are still subjected to DNA damage. Some types of damage cannot be corrected without the import of homologous DNA that can be used as template for recombinational repair of the damage. Thus, the reason that competence for natural transformation has not been lost in *S. thermophilus* is probably because this mechanism is needed for DNA repair. In *S. pneumoniae*, competent cells that are immune to their own lytic toxin acquire homologous DNA by lysing their non-competent sister cells or members of closely related species. However, the genome of *S. thermophilus* does not encode any homologues of the ComM immunity protein, and *S. thermophilus* must therefore protect itself against CbpD-St by some other mechanism. This mechanism does not seem to be competence induced as more than 30% of cells in a competent population are lysed in the presence of DTT. Perhaps the rapid inactivation of CbpD-St by H_2O_2 functions as the protective mechanism that ensures that only a fraction of the cells are lysed. Members of this fraction could then serve as DNA-donors that supply the competent survivors with templates for recombinational repair.

The *S. thermophilus* strains used in milk fermentation today have probably been continuously propagated in this protected environment for thousands of years (Bolotin *et al.*, 2004). Adaption to this new habitat must have made a number of gene products redundant. Some of these have been lost over the years, but some might have acquired new functions. It is therefore possible that CbpD-St and CbpD-Sp serve different functions in their respective hosts. The fact that CbpD-St influences transformability of *S. thermophilus* suggests that it might have evolved to make uptake of extracellular DNA more efficient during competence. How DNA polymers are translocated across the peptidoglycan layer has not been fully elucidated. However, it is known that the type IV-like pseudopili expressed during competence is essential for uptake of DNA (Dubnau, 1999; Chen *et al.*, 2004; Peterson *et al.*, 2004). These pili are retractable and it is therefore possible that DNA attaches itself to the pili, which subsequently pulls the DNA through the peptidoglycan layer (Craig *et al.*, 2004; Craig *et al.*, 2006). The role of CbpD-St could be to make the peptidoglycan layer more permeable to the pili and/or DNA polymers by introducing specific cuts in the oligopeptide cross-links.

In conclusion, more research is needed to discriminate between the two proposed models. One way to proceed might be to investigate the properties and function of the CbpD

proteins from *S. vestibularis* and *S. salivarius*. These oral commensals are the closest known non-domesticated relatives of *S. thermophilus*. If CbpD-St and the CbpDs produced by *S. vestibularis* and *S. salivarius* display very different properties, it is reasonable to assume that CbpD-St has lost its original function and evolved to serve a new function in the dairy habitat.

Peptide-regulated gene depletion system developed for use in *Streptococcus pneumoniae*

While the current study was in progress, the signal transduction pathway controlling ComX expression in *S. thermophilus* was elucidated (Gardan *et al.*, 2009; Fontaine *et al.*, 2010). Similarly to *S. pneumoniae* and other mitis group streptococci, competence development in *S. thermophilus* turned out to be controlled by a quorum-sensing-like mechanism. Apart from this, the ComABCDE and ComRS pathways used by *S. pneumoniae* and *S. thermophilus*, respectively, are completely unrelated. In fact, no close homologues of the ComRS proteins are present in the genome of *S. pneumoniae*. This made us realize that the ComRS pathway could be developed as a tool for peptide-regulated gene expression and gene depletion studies in *S. pneumoniae.* A well-functioning and easy-to-use gene depletion system for the study of essential pneumococcal genes would be especially useful. More than a hundred essential genes have been identified in this species (Thanassi *et al.*, 2002) and many of their products are involved in cellular processes that are poorly understood, e.g. cell division and cell-wall biosynthesis. These processes represent an Achilles' heel of bacteria, and will be the target of future antibiotics.

In the model of the ComRS pathway outlined by Fontaine *et al.* (2010) (Fig. 5), the ComS* signalling peptide is taken up by the Ami oligopeptide transporter. Evidence demonstrating that the Ami transporter is an essential component of the pathway had been reported earlier by Gardan and colleagues (2009). In the proposed model, internalized ComS* is postulated to bind and activate ComR. Evidence for this is not provided, but is based on the analogy to related systems that are better characterized (Shi *et al.*, 2005; Declerck *et al.*, 2007). Furthermore, Fontaine *et al*. (2010) assume that ComR binds to the *comX* promoter and activates transcription of the *comX* gene. Although this is a reasonable assumption, it is also possible that ComR activates ComX expression indirectly via another transcriptional activator. However, in order to succeed with our plan to develop a ComS*-regulated gene depletion system for the use in *S. pneumoniae*, the model proposed by Fontaine and co-

workers would have to be correct. We started by introducing the *comR* gene including its promoter into a neutral location in the *S. pneumoniae* genome. Next, the firefly luciferase gene driven by the *S. thermophilus comX* promoter was inserted at another neutral location resulting in the strain SPH126 (for details see Paper III). The *luc* gene is a very convenient reporter to use, as its activity can be measured continuously while cultivating the bacteria in a luminometer. This would allow us to determine whether addition of ComS* to the growth medium induces transcription from the *comX* promoter, and to measure how different concentrations of ComS* affects the transcription rate. *S. pneumoniae* possesses an Ami oligopeptide transporter of its own, and we therefore hoped that the native Ami transporter would be able to mediate efficient uptake of the synthetic ComS* peptide. Strain SPH126 was tested by growing it in a 96-well Corning NBS plate with a clear bottom at 37°C inside a Fluostar Optima luminometer in the presence and absence of ComS*. In the cultures subjected to ComS* (0-16 µM) light emission started to rise about 10 minutes post-induction and continued to increase for about 1 hour. This result proves that the model of Fontaine *et al.* is correct (Fig. 5), and also shows that the native Ami transporter is able to mediate import of ComS*.

Maximum light emission, which was obtained when SPH126 cultures were treated with 2 µM ComS*, appeared not to be very strong, and we therefore set out to see if it was possible to obtain higher expression of the *luc* reporter. We suspected that the oligopeptide-binding protein of the pneumococcal Ami transporter might not recognize ComS* very well. This would result in a low intracellular concentration of ComS* that would be rate limiting for *luc* expression. We therefore introduced the *S. thermophilus* oligopeptide-binding protein AmiA3 under control of its own promoter into the genome of the SPH126 strain. This did not make any difference, as the resulting mutant produced the same amount of light as the parental strain. The most likely explanation for this negative result is that the pneumococcal Ami transporter functions adequately. However, it is also possible that introduction of the "foreign" AmiA3 oligopeptide-binding protein did not contribute to increased uptake of ComS* because it was unable to function well in conjunction with the four integral membrane components (AmiCDEF) of the native pneumococcal Ami transporter. Another reason for the modest *luc* expression obtained with the SPH126 strain might be suboptimal expression of ComR. We therefore inserted a relatively strong synthetic constitutive promoter called P1 upstream of ComR in the SPH126 strain. The resulting strain SPH130 emitted about five-fold more light, when subjected to 2 µM ComS*, than the parental SPH126 strain. Based on our

long experience with the *luc* reporter system we knew that this level of light production is only reached with quite strong promoters. Furthermore, by reducing the concentration of the ComS* inducer, the strength of the *comX* promoter could be gradually reduced in a controlled manner (Paper III, Fig. 2a and b). In the absence of ComS*, no light emission above background levels could be detected. These results were very promising, and we therefore wanted to test our gene depletion system on a real problem.

It has been shown previously that genes required for teichoic acid production in *S. pneumoniae* cannot be deleted, demonstrating that the pneumococcus cannot survive without teichoic acid in its cell envelope (Zhang *et al.*, 1999; Baur *et al.*, 2009). The type of teichoic acid produced by *S. pneumoniae* and several other species in the mitis phylogenetic group is complex and unusual. The wall teichoic acid (WTA) and lipoteichoic acid (LTA), often called C-polysaccharide and F-polysaccharide, respectively, have identical structures except for the part that anchors LTA to the membrane (Fig. 7) (Yother *et al.*, 1998). One of the unusual features is that both WTA and LTA are decorated by phosphorylcholine residues, which serve as an attachment site for surface proteins containing choline-binding domains (Sánches-Puelles *et al.*, 1990; Fischer *et al.*, 1993). Each repeat unit (see Fig. 7) is translocated across the cytoplasmic membrane by a transporter named TacF, and polymerized at the outer surface of the cytoplasmic membrane by an unknown enzyme (Rahman *et al.*, 2009). TacF, which is an essential protein in *S. pneumoniae*, has strict specificity for teichoic acid subunits containing choline (Damjanovic *et al.*, 2007). As a consequence, subunits lacking choline will not be transported, and the cell will die. The enzymes that attach phosphorylcholine to the teichoic acid subunits are LicD1 and LicD2. The genes encoding these proteins are located on the same transcriptional unit as the *tacF* gene (*tacF-licD1-licD2*). It has been shown previously that whereas *licD2* can be deleted, it is not possible to delete both *lic* genes at the same time (Zhang *et al.*, 1999). Studies of the precise function of teichoic acids in *S. pneumoniae* have been hampered by the absence of mutants lacking this polymer. We therefore chose to test our depletion system by constructing a *licD1/licD2* double-mutant in which the level of LicD1 expression could be gradually reduced. The SPH135 strain was constructed by first inserting the *licD1* gene behind the *comX* promoter, and then deleting the *licD1* and *licD2* genes in the presence of ComS* (see Paper III for details). When the SPH135

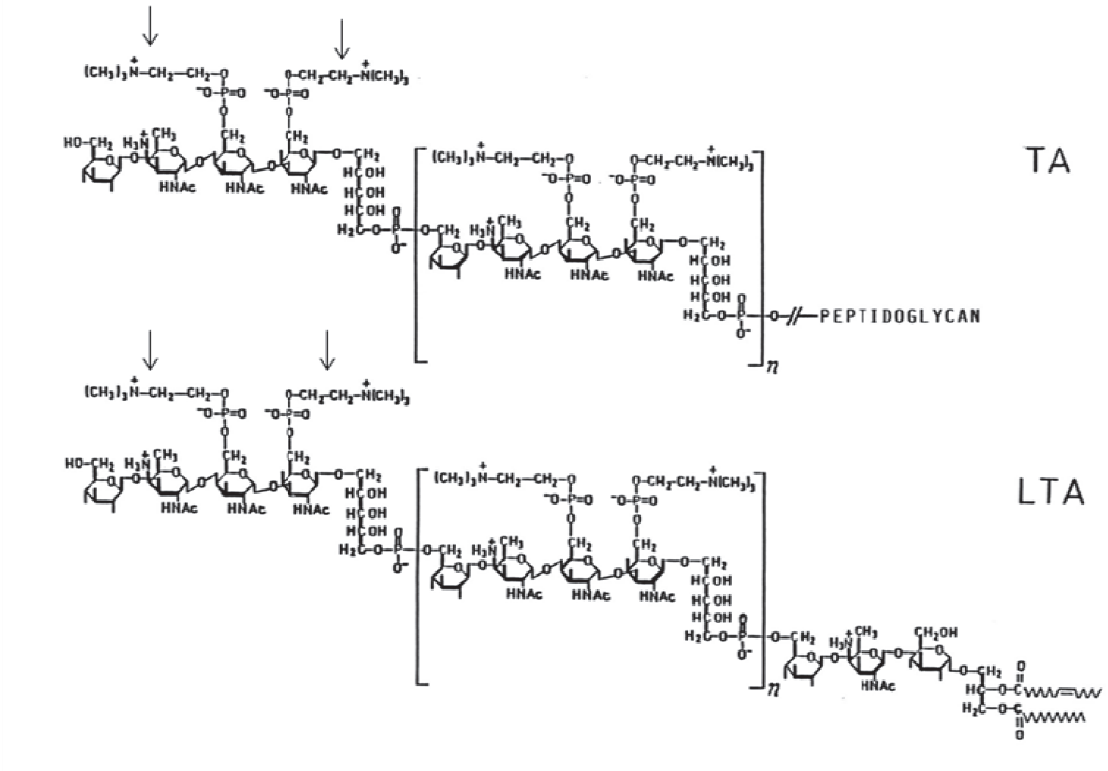

Figure 7: Structure of wall teichoic acid (TA) and lipoteichoic acid (LTA) from S. *pneumoniae.* Arrows indicate the positions of the phosphoryl choline residues. Reprinted from Yother *et al*. (1998) with permission from ASM.

strain was shifted from ComS*-containing (2 µM) to a ComS*-free medium it took 7-8 hours before the cultures stopped growing. However, reduced growth rate could be observed after about 5 hours (Paper III, Fig. 4a and b). We wondered if this relative long response time could be shortened if the culture was grown at a lower ComS* concentration before it was shifted to ComS*-free medium. It turned out that 0.02 µM ComS* was sufficient to give normal growth, and that the response time for cells transferred from this ComS* concentration to ComS*-free medium was reduced to 3-4 hours. This result suggests that ComS* is metabolized slowly by the pneumococcus. We also speculated that the *comX* promoter might be leaky, and that low-level constitutive expression from this promoter could add to the relative long response time. To address this question we performed transcriptional analyses on the SPH130 strain, which contains the *luc* gene inserted behind the *comX* promoter. By using RT-PCR we were able to show that the level of *luc* transcripts in uninduced cells is 10-30 times higher than the level of *cbpD* transcripts. As *cbpD* belongs to the late competence genes, the number of *cbpD* transcripts in non-competent cells is probably close to zero.

Nevertheless, the results showed that there is a low-level ComS*-independent background transcription originating from the *comX* promoter. This leakiness will only constitute a problem, however, if the gene selected for depletion studies is naturally expressed at a very low level in the pneumococcal cell.

Microscopic inspection of pneumococci depleted for teichoic acid revealed that they were larger than normal and had a grossly deformed elongated shape (Paper III, Fig. 4c). The elongated shape strongly indicates that the cells struggle to divide. Since undecaprenyl diphosphate is the carrier molecule for teichoic acid precursors as well as peptidoglycan precursors, it is possible that LicD1 depletion could also affect cell-wall synthesis. Inhibition of TacF could trap undecaprenyl-linked choline-free teichoic acid precursors inside the cytoplasmic membrane, thereby draining the pool of undecaprenyl diphosphate needed for transport of peptidoglycan precursors. Judging from the fact that the LicD1 depleted cells are much larger than normal, it seems unlikely that their cell wall synthesis is severely affected. Consequently, the observed abnormalities are probably due to stress caused by sub-optimal levels of teichoic acids in the cell wall of the pneumococci.

The results obtained with the SPH135 mutant show that our ComRS-based gene depletion system is functioning as intended. It has the potential of becoming a very valuable tool for the study of essential genes in *S. pneumoniae* and other Gram-positive bacteria lacking close homologues of ComRS. Two other gene depletion systems have been used successfully in *S. pneumoniae*. One is based on a pneumococcal fucose-regulated promoter (Chan *et al.*, 2003), while the other builds on a Zn^{2+}-inducible promoter (Kloosterman *et al.*, 2008; Eberhardt *et al.*, 2009). The latter system functions by using a relative high concentration of Zn^{2+} to drive ectopic expression of the selected gene, followed by removal of Zn^{2+} from the medium to initiate depletion of the gene. Zinc plays an important role in the physiology of pneumococci and most other bacteria (Dintilhac *et al.*, 1997; Kloosterman *et al.*, 2007; Ogunniyi *et al.*, 2009). Unphysiological levels of this divalent cation in the growth medium could therefore have unintended effects that influence the result of a depletion experiment. An advantage of the ComRS over the fucose- and zinc-based systems is that it probably has no impact whatsoever on the biology of *S. pneumoniae*. This assumption is based in the fact that the genome of *S. pneumoniae* encodes no close homologs of the ComRS proteins. However, in the future, microarray analyses should be carried out to control that

ComS*-induced pneumococci expressing ComR do not have an altered gene expression profile compared to wild-type cells.

Concluding remarks

Since papers I, II and III deal with different aspects of natural transformation in *S. thermophilus*, I chose to discuss them separately. The findings presented in paper I and II show that many features of the natural transformation machinery are shared between *S. thermophilus* and the paradigm organism *S. pneumoniae*. In both species accumulation of ComX under conditions inappropriate for competence development is prevented by their Clp systems. Nevertheless, as the key component of this mechanism in *S. thermophilus*, ClpC, does not appear to be important in *S. pneumoniae*, the mechanisms cannot be identical. Furthermore, similarly to the pneumococcus, competent *S. thermophilus* cells express a CbpD-like murein hydrolase that is able to lyse susceptible sister cells. At present, however, it is unclear whether CbpD-St and CbpD-Sp serve the same or different functions in their respective species. Interestingly, even though most genes encoding proteins involved in DNA uptake, processing and recombination are conserved between *S. thermophilus* and *S. pneumoniae*, this is not the case for the signal transduction pathways controlling these core competence genes. This gave us the opportunity to develop a titratable gene depletion system that is likely to become a valuable tool for researchers investigating the function of essential genes in *S. pneumoniae* and other Gram-positive bacteria.

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Paper I

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ClpC acts as a negative regulator of competence in *Streptococcus thermophilus*

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The alternative sigma factor ComX is a key regulator of natural transformation in members of the genus *Streptococcus*. ComX controls expression of the late competence genes, which are essential for DNA binding, uptake and recombination. In *Streptococcus pneumoniae*, it has been demonstrated that ComX is degraded by ClpEP at the end of the competence period. In the present study we show that a different Clp protease complex, ClpCP, contributes to ComX degradation in *Streptococcus thermophilus*. Mutant strains lacking the ClpC chaperone displayed significantly increased transformability compared with the wild-type strain under conditions where ComX was expressed at relatively low levels. At higher expression levels, ClpCP appears to become saturated and unable to prevent the accumulation of ComX. Together, our results suggest that the role of ClpC is to mediate degradation of ComX when the sigma factor is produced in low amounts, i.e. when the environmental stimulus promoting competence development is weak. This would prevent *S. thermophilus* from developing the competent state at an inappropriate time and/or place.

INTRODUCTION

Recently, it was discovered that *Streptococcus thermophilus*, an economically important bacterium for the dairy industry, is competent for natural genetic transformation (Blomqvist *et al.*, 2006b; Gardan *et al.*, 2009; Fontaine *et al.*, 2010). It was found that the alternative sigma factor ComX controls the core competence genes, i.e. all genes needed for DNA binding, uptake and recombination (Blomqvist *et al.*, 2006b). This is in accordance with the situation in *Streptococcus pneumoniae*, where expression of the so-called late competence genes directly depends on ComX (Lee & Morrison, 1999). In *S. pneumoniae comX* expression is regulated by a quorum-sensing-like system encoded by the *comCDE* genes (Håvarstein *et al.*, 1995; Pestova *et al.*, 1996). The system consists of a secreted competence-stimulating peptide (CSP) which is encoded by the *comC* gene, its membrane-anchored histidine kinase receptor ComD and the cognate response regulator ComE (for review, see Johnsborg & Håvarstein, 2009). The genome of *S. thermophilus* contains a signal-transduction system of the ComCDE-type (BlpC and BlpRH) as well. However, this system does not control competence development, but regulates the production of several different bacteriocins and their immunity proteins (Fontaine *et al.*, 2007). Regulation of competence development in *S. thermophilus* is based on a different quorum-sensing mechanism involving the oligopeptide pheromone ComS and the transcriptional activator ComR (Fontaine *et al.*, 2010). Mature ComS is derived from the C-terminal end of a 24 aa ribosomally synthesized precursor. It is not known how ComS is processed and externalized, but the mature peptide (8 aa) is imported through the Ami oligopeptide transport system. Inside the cell mature ComS presumably binds to ComR, resulting in an active form of this transcription factor that drives transcription of the *comX* gene (Fontaine *et al.*, 2010). The environmental cues or growth conditions triggering ComS production and spontaneous competence development in *S. thermophilus* have not been identified.

The evolutionarily conserved serine protease, ClpP, which monitors protein quality in general and controls the stability and activity of certain regulatory proteins, has been shown to act as a negative regulator of competence gene expression in *S. pneumoniae* (Chastanet *et al.*, 2001; Robertson *et al.*, 2002). The ClpP oligomer consists of 14 subunits arranged in two heptameric rings resembling a barrel. To gain proteolytic activity, the barrel-shaped proteolytic core must form a stable complex with hexameric rings of ATP-dependent chaperones termed Clp ATPases. It is the members of this protein family and not ClpP, that confer substrate specificity to the functional chaperone/protease complex (Frees *et al.*, 2007; Kress *et al.*, 2009). In *S. pneumoniae*, ComX remains stable after competence induction in a mutant lacking ClpP and/or ClpE. This indicates that ClpE specifically recognizes ComX and associates with the ClpP proteolytic domain to degrade the alternative sigma factor (Sung & Morrison, 2005; Piotrowski *et al.*, 2009a). To investigate whether ComX is

Abbreviation: RLU, relative light units.

subjected to regulation by the Clp system in *S. thermophilus* LMG18311, we deleted several of the *clp* genes in this strain. Interestingly, we found that ClpC-deficient strains displayed increased transformability compared with ClpC-proficient strains under certain conditions. This result shows that ClpC acts as a negative regulator of competence in *S. thermophilus*.

METHODS

Bacterial strains and growth media. The following media were used to cultivate *S. thermophilus* LMG 18311 and mutants derived from this strain: Todd–Hewitt broth (TH) supplemented with 0.8 % glucose (THG), Hogg–Jago glucose broth (HJG) consisting of 3 % tryptone, 1 % yeast extract, 0.2 % beef extract, 0.5 % KH_2PO_4 and 0.5 % glucose, HJG supplemented with 0.5 % lactose (HJGL), HJGLS, which is HJGL supplemented with 0.4 M D-sorbitol, and C medium (Lacks & Hotchkiss, 1960). Agar plates were prepared by adding 1.5 % (w/v) agar to the media. All incubations were carried out at 37 °C and all optical density measurements of bacterial cultures were performed spectrophotometrically at 492 or 550 nm.

Electroporation of *S. thermophilus* LMG 18311. Cultures grown overnight at 37 °C were diluted 100-fold in HJG prewarmed to 37 °C and incubated at this temperature until OD_{550} reached 0.3. Cultures (50 ml aliquots) were then diluted 1 : 1 in HJG (37 °C) containing 20 % glycine. After further incubation at 37 °C for 1 h, cells were harvested by centrifugation at 5000 ***g*** for 5 min at 4 °C. Next the cells were washed with 1 vol ice-cold electroporation buffer [5 mM $KHPO_4$, 0.4 M D-sorbitol, 10 % (v/v) glycerol; pH 4.5] and pelleted by centrifugation at 5000 ***g*** for 10 min at 4 °C. The washing step was repeated once. Finally, pelleted cells were resuspended in 2 ml ice-cold electroporation buffer, divided into aliquots and frozen in liquid nitrogen. Electrocompetent cells were stored at −80 °C. Plasmid DNA electroporation was performed with a Bio-Rad MicroPulser unit as described previously (Blomqvist *et al.*, 2006b).

Natural transformation of *S. thermophilus* LMG 18311. *S. thermophilus* LMG 18311 cells harbouring the pXL plasmid were grown overnight at 37 °C in THG supplemented with 2 μg erythromycin ml^{-1}. The next day the culture was diluted to OD_{550} 0.05 in the same medium prewarmed to 37 °C and 250 ng peptide pheromone (STP) ml^{-1}, which controls bacteriocin production in *S. thermophilus* LMG 18311 (NH_2-SGWMDYINGFLKGFGGQRTLPT-KDYNIPQV-COOH) was immediately added. When OD_{550} reached 0.2, culture samples (1 ml) were transferred to Eppendorf tubes containing transforming DNA and further incubated for 2 h at 37 °C. Samples were then put on ice, serially diluted and spread on HJGL agar plates containing the appropriate antibiotic (150 μg spectinomycin ml^{-1} or 100 μg kanamycin ml^{-1}). An identical protocol was followed when competence in *S. thermophilus* was induced by the native pheromone, ComS (NH_2-LPYFAGCL-COOH), instead of STP. In this case 900 ng ComS ml^{-1} was added to the culture. In the transformation experiments presented in Table 2, 3 μg ml^{-1} of a plasmid (pCR2.1-TOPO) carrying a *comEC* gene disruption cassette (Blomqvist *et al.*, 2006b) was used as transforming DNA. Curing of the pXL plasmid was performed by cultivating transformants in antibiotic-free medium for about 100 generations.

Construction of plasmids used for reporter assays and Western analysis. The construction of the plasmids used for the luciferase reporter assays, pXL, pEAP and pXP, has been described previously (Blomqvist *et al.*, 2006b). To make the pXL-His plasmid we employed the comX_His_F and comX_His_R primers to amplify a fragment corresponding to the bacteriocin promoter and the adjacent *comX* gene. The pXL plasmid was used as template. To facilitate subsequent cloning, *Bam*HI and *Eco*RV restriction sites were engineered into the 5′-ends of comX_His_F and comX_His_R, respectively. In addition, a C-terminal 6× His-tag was inserted into the comX_His_R primer after the last amino acid of the ComX protein. The PCR fragment obtained with the comX_His_F and comX_His_R primers was first cloned into the pCR 2.1-TOPO vector (Invitrogen). Next the fragment was excised from this vector by cleavage with *Bam*HI and *Eco*RV and ligated into the pEAP plasmid precleaved with *Bam*HI and *Sma*I. The resulting plasmid was designated pXL-His. All PCRs were carried out with the Phusion high-fidelity DNA polymerase (Finnzymes) and the final constructs were checked by complete sequencing.

Construction of mutants. The various target genes were disrupted by replacing the respective genes with a spectinomycin (*clpC*, *clpE*, *clpL* and *clpX*) or kanamycin (*clpP* and *comX*) resistance cassette. This was achieved by double-crossover homologous recombination using a PCR fragment consisting of the antibiotic resistance gene flanked by ~1000 bp DNA fragments corresponding to the flanking 5′ and 3′ regions of the target gene. In the first step the primer pairs Spec-F(A)/Spec-R or Kana-F/Kana-R were used to amplify the spectinomycin or kanamycin resistance marker, respectively (Table 1). Plasmid pR412 was the source of the synthetic spectinomycin resistance cassette (Martin *et al.*, 2000), while the kanamycin resistance gene was amplified from the pFW13 vector (Podbielski *et al.*, 1996). Next, PCR fragments corresponding to the flanking regions of the target genes were amplified by using the appropriate primer pairs (Table 1). Finally, the three fragments constituting each gene disruption cassette were stepwise joined by overlap-extension PCR, in each case by using the appropriate external primers. The resulting gene disruption cassette was purified with a PCR purification kit obtained from Macherey-Nagel and used directly to transform *S. thermophilus* cells by natural transformation.

To construct a T2 derivative with a wild-type *clpC* allele, the Up_clpC_F and Down_clpC_R primers were used in PCR to amplify a fragment consisting of the wild-type *clpC* gene plus flanking regions of about 1000 bp. The resulting PCR fragment was used to transform T2 cells harbouring the pXL plasmid. After overnight growth, 95 single colonies were picked at random from HJGL agar plates and transferred into individual wells of a microtitre plate containing THG medium and D-luciferin (see luciferase reporter assay below). After growth in an Optima FLUOstar luminometer at 37 °C overnight, one clone was identified that displayed a light emission curve that was different from all the others. Sequence determination of the entire *clpC* gene of this clone, designated T2-R, revealed that the mutant gene had been replaced by the wild-type allele.

Luciferase reporter assays. Detection of luciferase activity was performed essentially as described by Blomqvist *et al.* (2006b). Briefly, cultures grown overnight in THG at 37 °C were diluted to OD_{492} 0.05 in prewarmed THG and further incubated at 37 °C until they reached OD_{492} 0.5. Then, cultures were diluted 10-fold in the same medium and 280 μl diluted culture was immediately mixed with firefly D-luciferin (20 μl 10 mM D-luciferin solution in THG). If appropriate, STP was added to a final concentration of 250 ng ml^{-1}. Next, samples were transferred into a 96-well Corning NBS plate with a clear bottom. The plate was incubated in an Optima FLUOstar luminometer at 37 °C for 8 h. Luciferin–luciferase luminescence and OD_{492} were measured automatically by the luminometer at 10 min intervals.

Detection of ComX-His by Western analysis. The pXL-His plasmid was electroporated into *S. thermophilus* LMG 18311 and a Δ*clpC* mutant strain that had been cured from the pXL plasmid. Cultures of these strains were grown overnight at 37 °C in THG

Table 1. Primers used in this study

Primer	Gene	Primer sequence (5′–3′)
Up_clpC_F	*clpC*	CAAGCTTTTGACCCCCACTA
Up_clpC_R	*clpC*	CTCGAGGTCGACGGTATCGCGCATCTTCGCCTAAAACAT
Down_clpC_F	*clpC*	ATTGGATCCATTCCGCGTCAACCGTAAAGCAGGCGTAAAAG
Down_clpC_R	*clpC*	GAAATCGGAGGCACTGAGAG
Up_clpL_F	*clpL*	GGTTTATTCTTGTCTAACTCAAAACCA
Up_clpL_R	*clpL*	CTCGAGGTCGACGGTATCGGACTTTCCTTGGGTGCTACG
Down_clpL_F	*clpL*	ATTGGATCCATTCCGCGTCAATTGCTCTTGGTAGAAGGCTGA
Down_clpL_R	*clpL*	AGGTAGCGACCAAACGCTAT
Up_clpE_F	*clpE*	CCGCCAGGTTATGGAAGTCT
Up_clpE_R	*clpE*	CTCGAGGTCGACGGTATCGTCCAACATTTGGTGACAAGG
Down_clpE_F	*clpE*	ATTGGATCCATTCCGCGTCAATTTCTATCTGAGTTGGGAAAGACC
Down_clpE_R	*clpE*	CCTCTTCGCCCATAGAAACC
Up_clpX_F	*clpX*	ACGCTTGGTCCTCAATGTTC
Up_clpX_R	*clpX*	CTCGAGGTCGACGGTATCGGGTGCCATAGAAGGCATGAA
Down_clpX_F	*clpX*	ATTGGATCCATTCCGCGTCAAACCTGAAGTTGCCCTAGCAG
Down_clpX_R	*clpX*	GATCCCGTTACTGGCATAGC
Up_clpP_F	*clpP*	GGCTATGGTTTAGTAACAGGTAA
Up_clpP_R	*clpP*	AAGCTTAAGATCTAGAGCTCGAACCGGAATCATATGAATTCTCCTT
Down_clpP_F	*clpP*	AGCATGCATATGCATCCGGAGTGATCAAGCAATAGACAATCTTGTTTTACAT
Down_clpP_R	*clpP*	CCATTCTGCTCTGACTTACCAT
ComX_His_F	*comX-His*	ATTAGGATCCTTCAAGGTCTAGTCCTCTCTTTTATGACG
ComX_His_R	*comX-His*	AATTAGATATCTCAATGATGATGATGATGGTCTTCT TCATTACATGGATCAAAGTC
Kana-F	*kan*	ATCCTCGAGCTCTAGATCTTAAGCTT
Kana-R	*kan*	ACTCCGGATGCATATGCATGCT
Spec-F(A)	*spc*	CGATACCGTCGACCTCGAG
Spec-R	*spc*	TTGACGCGGAATGGATCCAAT
Up_comX_F	*comX*	GACGTAGTAGAGTTGGCGTTCC
Up_comX_R	*comX*	AAGCTTAAGATCTAGAGCTCGAGGATAAGCGAGATAGAGTCAAAACCA
Down_comX_F	*comX*	AGCATGCATATGCATCCGGAGTTCCAGTAATTCTTGGAAGGTCAA
Down_comX_R	*comX*	TCTCCAATTTAATAACAAGAAAAACG

supplemented with 2 μg erythromycin ml^{-1}. The next morning they were diluted to OD_{492} 0.05 in the same medium, immediately induced by adding 250 ng STP ml^{-1} and further incubated at the same temperature in a water bath. When OD_{492} 0.2, 0.4 and 0.6 was reached, samples (10 ml) were withdrawn and the bacteria were pelleted by centrifugation. Pellets from these samples were resuspended in 100, 200 and 300 μl Laemmli sample buffer, respectively, and stored at −80 °C. After heating to 95 °C for 5 min, samples (35 μl) were loaded onto 15 % SDS-PAGE precast gels (Bio-Rad), separated and transferred to a PVDF membrane. His-tagged ComX was detected using the Pierce SuperSignal West Pico HisProbe kit according to the manufacturer's instructions.

RESULTS

Altered transcription of late competence genes in *clp*-deletion mutants

In addition to the *clpP* gene, the genes encoding the Clp ATP-ases ClpC, ClpE, ClpL and ClpX are present in the genome of *S. thermophilus*. To determine whether any of these Clp proteins are involved in regulating expression of the late competence genes we set out to construct deletion mutants corresponding to each *clp* gene. We did not succeed in making a Δ*clpX* mutant, which indicates that *clpX* might be an essential gene. Similarly, when trying to knock out the *clpP* gene we obtained only a few transformants that grew very poorly. In contrast, Δ*clpC*, Δ*clpE* and Δ*clpL* mutants were readily obtained and displayed growth characteristics similar to those of the wild-type strain. To disrupt the *clp* genes, we used overlap extension PCR to generate a DNA fragment consisting of an antibiotic selection marker fused to ~1000 bp flanking sequences amplified from the immediate upstream and downstream regions of the genes to be deleted. The resulting fragment was taken up by competent *S. thermophilus* LMG 18311 cells and integrated into the genome of recipients by double-crossover homologous recombination.

Two different methods for inducing the competent state in *S. thermophilus* were used in this study: a procedure that relies on ectopic expression of ComX from the pXL plasmid (Blomqvist *et al.*, 2006b) and activation of ComX expression by addition of the newly identified *S. thermophilus* competence pheromone ComS (Fontaine *et al.*, 2010). The pXL plasmid, which is a derivative of the pTRKH2 plasmid, contains a *comX* gene driven by a bacteriocin promoter and a firefly luciferase gene (*luc*) fused to the *comEC* late gene promoter (Blomqvist *et al.*,

Table 2. Transformation efficiency of various strains and mutants of *S. thermophilus*

Plasmid	Pheromone	Medium	Transformation efficiency*
***S. thermophilus* LMG 18311**			
pXL	None	THG	0
pXL	STP	THG	0.048 ± 0.006
None	None	THG	0
None	ComS	THG	0.74 ± 0.05
None	None	C	0
None	ComS	C	0.66 ± 0.1
S. thermophilus* LMG 18311 Δ*clpC			
pXL	None	THG	0.002 ± 0.0007
pXL	STP	THG	0.046 ± 0.009
None	None	THG	0
None	ComS	THG	0.05 ± 0.03
None	None	C	0.02 ± 0.008
None	ComS	C	0.30 ± 0.15
***S. thermophilus* LMG 18311-T2**			
pXL	None	THG	0.089 ± 0.01
pXL	STP	THG	1.75 ± 0.5
None	None	THG	0
None	ComS	THG	0.074 ± 0.02
None	None	C	0.005 ± 0.003
None	ComS	C	2.2 ± 1.0
***S. thermophilus* LMG 18311-T2-R**			
pXL	None	THG	$2 \times 10^{-4} \pm 3 \times 10^{-5}$
pXL	STP	THG	0.071 ± 0.011

*Transformants (c.f.u.)/total number of bacteria (c.f.u.) $\times$ 100 $\pm$ SEM. Individual transformation experiments were repeated between three and seven times.

2006b). When the peptide pheromone (STP), which controls bacteriocin production in *S. thermophilus* LMG 18311, is added to bacteria carrying the pXL plasmid, expression of ComX is induced. Since the bacteriocin promoter is leaky, low level ComX expression can be observed even in the absence of the STP peptide (Blomqvist *et al.*, 2006a, b). As ComX specifically directs transcription from late gene promoters, the intracellular level of this sigma factor is reflected by the level of luciferase activity. To determine whether deletion of *clpC*, *clpE* or *clpL* affected light production, culture samples of these mutants were grown in 96-well Corning NBS plates at 37 °C inside an Optima FLUOstar luminometer as described previously (Blomqvist *et al.*, 2006b). The results showed that an uninduced pXL strain lacking *clpC* produced much more light than the corresponding uninduced wild-type strain (Fig. 1a). Intriguingly, STP-induced and uninduced cultures of this *clpC* mutant displayed identical levels of luciferase activity. Deletion of the *clpE* gene resulted in increased reporter activity in both induced and uninduced cultures, whereas the level of light production in a Δ*clpL* mutant remained largely unaltered compared with the wild-type (Fig. 1b). As deletion of *clpC* resulted in a dramatic increase in light emission in uninduced cultures, i.e. under conditions where the intracellular levels of ComX are low, we decided to focus on ClpC and its role in the regulation of natural transformation in *S. thermophilus* LMG 18311.

Deletion of *clpC* strongly upregulates transcription of late competence genes without affecting the level of *comX* transcription

The strong increase in late gene transcription observed in a Δ*clpC* mutant indicates that ClpC directly or indirectly influences the level or activity of ComX in the cell. To further elucidate these matters we took advantage of two previously constructed reporter plasmids (Blomqvist *et al.*, 2006b). In one of these, pXP, the luciferase gene is driven by the *comX* promoter, while in the other, pEAP, the ComEC late gene promoter is placed upstream of the *luc* reporter (Blomqvist *et al.*, 2006b). None of these plasmids contains the STP-inducible *comX* gene carried on the pXL plasmid described above. When the pXP plasmid was introduced into *S. thermophilus* wild-type cells and a Δ*clpC* mutant by electroporation, no difference in reporter activity was observed between the two strains (Fig. 1d). These results show that ClpC does not affect the level of ComX expression in *S. thermophilus* cells growing in THG. Interestingly, however, when the pEAP plasmid was introduced into wild-type and Δ*clpC* cells, light was produced only by the cells lacking ClpC (Fig. 1c). To make sure that the emitted light results from ComX-driven transcription of the pEAP-located luciferase gene, the *comX* gene was deleted from the chromosome of Δ*clpC* cells carrying the pEAP plasmid. Luciferase reporter assays showed that no light was produced by the resulting *comX*-deficient strain (results not shown). This proves that ClpC acts through ComX and not through another regulatory protein that in theory might have affected transcription of the late competence genes. Together, these results indicate that ClpC regulates the level or activity of ComX post-translationally.

The *clpC* deletion mutant develops the competent state spontaneously when grown in C medium

To determine whether the increased level of late gene expression observed in the Δ*clpC* mutant has a positive effect on the transformability of this mutant compared with the wild-type strain, a number of transformation assays were performed (see Table 2). A pCR 2.1-TOPO plasmid carrying a kanamycin resistance gene flanked at both ends by about 1000 bp DNA originating from the upstream and downstream regions of the *S. thermophilus comEC* gene was used as donor DNA in these transformation experiments. In THG medium, assays carried out with Δ*clpC* and wild-type strains carrying the pXL plasmid demonstrated that the Δ*clpC* strain transformed with the same efficiency as the corresponding wild-type strain when the strains were induced by STP. However, in contrast with

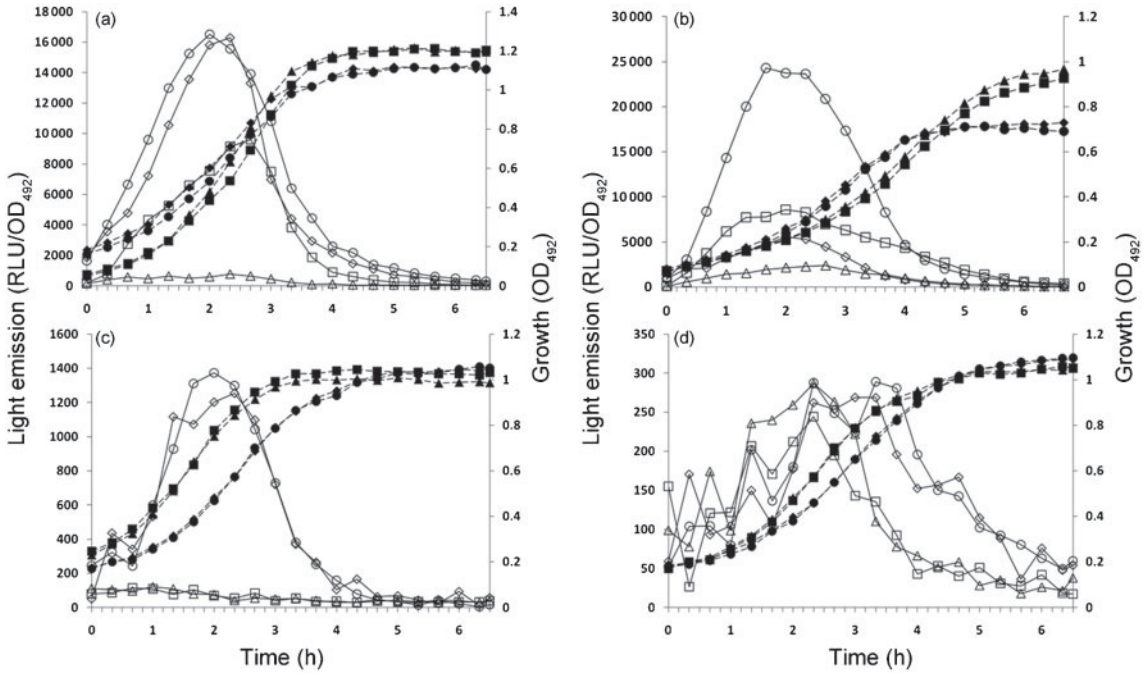

Fig. 1. Luciferase reporter assays that show the activity of the competence-specific promoters P_{comEC} and P_{comX} in wild-type (WT) and *clp* deletion mutants of *S. thermophilus.* Continuous lines and open symbols represent light emission [relative light units (RLU) divided by OD_{492}], while dashed lines and filled symbols represent growth curves. Cultures treated with STP received 250 ng ml^{-1} at time zero. (a) WT (squares, +STP; triangles, −STP) and Δ*clpC* mutant (circles, +STP; diamonds, −STP) carrying the pXL plasmid. pXL contains a *comX* gene driven by a leaky bacteriocin promoter that is inducible by the STP peptide pheromone. In addition the pXL plasmid contains a firefly luciferase gene (*luc*) fused to the *comEC* late gene promoter. (b) Δ*clpL* mutant (squares, +STP; triangles, −STP) and Δ*clpE* mutant (circles, +STP; diamonds, −STP) carrying the pXL plasmid. (c) WT (squares, +STP; triangles, −STP) and Δ*clpC* mutant (circles, +STP; diamonds, −STP) carrying the pEAP plasmid. The pEAP plasmid differs from the pXL plasmid in that it contains only a *luc* gene fused to the *comEC* late gene promoter. (d) WT (squares, +STP; triangles, −STP) and Δ*clpC* mutant (circles, +STP; diamonds, −STP) carrying the pXP plasmid. The pXP plasmid contains a *luc* gene fused to the *comX* promoter. These experiments were repeated at least three times with highly similar results.

the wild-type strain, the Δ*clpC* strain was also naturally slightly transformable without addition of STP, i.e. without STP-induced overexpression of ComX. This is due to the leaky bacteriocin promoter situated upstream of *comX*, which allows low-level ComX expression even in the absence of STP. In wild-type and Δ*clpC* strains lacking the pXL plasmid, transformation in THG medium was only observed in the presence of the ComS pheromone. No spontaneous transformation was detected under these conditions. Interestingly, however, a screening of different growth media revealed that a Δ*clpC* mutant lacking the pXL plasmid became spontaneously competent for natural transformation when grown in semi-synthetic C medium. No transformants were obtained when the corresponding wild-type strain was grown in this medium (Table 2).

Identification of a competence-up mutant

When testing different clones of the Δ*clpE* and Δ*clpL* mutants described above we noticed that in both cases two different phenotypes appeared. One variant corresponded to the Δ*clpE* and Δ*clpL* phenotypes described in Fig. 1(b), while the other was more similar to the Δ*clpC* phenotype (Fig. 1a). The characteristic phenotype of Δ*clpC* mutants containing the pXL plasmid is that there is no difference between the amount of light produced in the presence or absence of STP induction. The solution to this puzzle turned out to be that our stock of the *S. thermophilus* LMG 18311 strain was not homogeneous, but contained two variants with respect to transformability. In the presence of the pXL plasmid, the majority of the cells representing the wild-type (WT) transformed with significantly less efficiency than the other variant (T2). The two different phenotypes, WT and T2, were stable and did not change even after cultivation for hundreds of generations. All experiments presented in Fig. 1 are performed with WT or *clp* mutants of WT cells. As the phenotype of T2 cells closely resembled that of WT cells lacking a functional *clpC* gene (Fig. 2), we speculated that T2 cells might represent a natural *clpC* mutant. We therefore amplified the *clpC* gene from T2 cells by PCR and sequenced the resulting DNA fragment. Indeed, the results showed that the T2 *clpC* gene

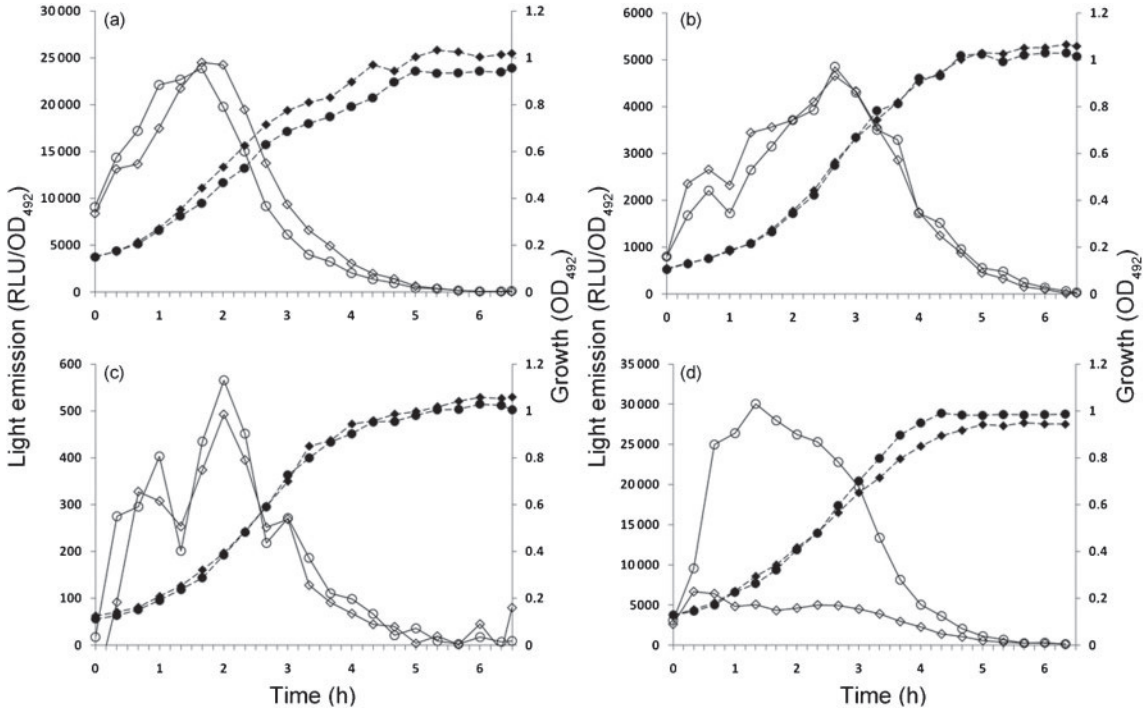

Fig. 2. Luciferase reporter assays that show the activity of the competence-specific promoters P_{comEC} and P_{comX} in a natural isolate of *S. thermophilus* termed T2. Continuous lines and open symbols represent light emission (RLU divided by OD_{492}), while dashed lines and filled symbols represent growth curves. Cultures treated with STP received 250 ng ml^{-1} at time zero. (a) T2 strain (circles, +STP; diamonds, −STP) carrying the pXL plasmid. pXL contains a *comX* gene driven by a leaky bacteriocin promoter that is inducible by the STP peptide pheromone. In addition the pXL plasmid contains a firefly luciferase gene (*luc*) fused to the *comEC* late gene promoter. (b) T2 strain (circles, +STP; diamonds, −STP) carrying the pEAP plasmid. The pEAP plasmid differs from the pXL plasmid in that it contains only a *luc* gene fused to the *comEC* late gene promoter. (c) T2 strain (circles, +STP; diamonds, −STP) carrying the pXP plasmid. The pXP plasmid contains a *luc* gene fused to the *comX* promoter. (d) T2-R strain (circles, +STP; diamonds, −STP). This strain is identical to the T2 strain except that it carries a repaired *clpC* gene. In the T2-R strain the $G_{1630}\rightarrow T_{1630}$ nonsense mutation identified in the *clpC* gene of the T2 strain has been back-mutated to the wild-type sequence. These experiments were repeated at least three times with highly similar results.

contains a point mutation ($G_{1630}\rightarrow T_{1630}$) resulting in the introduction of a stop codon at amino acid position 544. Consequently, the *clpC* gene product of the T2 strain will be shortened by 273 aa at the C-terminal end. The $G_{1630}\rightarrow T_{1630}$ point mutation was not introduced by the PCR used to generate the sequencing template, as the *cplC* gene was amplified from the T2 strain and sequenced several times with the same result. To determine whether the $G_{1630}\rightarrow T_{1630}$ point mutation is responsible for the particular phenotype of the T2 strain, we reintroduced a functional *clpC* gene into this strain by transformation with a PCR fragment consisting of the wild-type allele plus flanking regions of about 1000 bp. The resulting strain was termed T2-R. Interestingly, luciferase reporter assays carried out with the T2-R strain showed that it behaved differently from its parental strain (Fig. 1a, d). When induced with the STP pheromone, T2-R emitted roughly the same amount of light as the T2 strain. However, in uninduced cells, where ComX is expressed only at low levels, light emission in the T2-R cells was reduced about fivefold compared with the T2 cells. This result demonstrates that the $G_{1630}\rightarrow T_{1630}$ nonsense mutation strongly contributes to the high-level late gene expression observed in uninduced T2 cells. Compared with wild-type cells harbouring the pXL plasmid, the uninduced T2-R strain still produces at least fivefold more light (see Figs 1a and 2d). This indicates that the T2 strain carries additional mutations that together with the $G_{1630}\rightarrow T_{1630}$ point mutation give rise to the T2 phenotype.

Estimation of the transformation efficiencies of the WT Δ*clpC* mutant and the T2 strain showed that the transformation rate of the T2 strain is about 40-fold higher than that of the Δ*clpC* mutant when the strains are grown in plain THG medium and THG containing the STP pheromone. In THG medium supplemented with the ComS pheromone their transformation rates are similar, while the T2 strain transformed about sevenfold better in C medium containing ComS. However, when grown in C medium in the absence of ComS the Δ*clpC* mutant gives

rise to about four times more transformants than the T2 strain (Table 2). Comparison of the transformabilities of the T2 and T2-R strains shows that the T2 strain transforms about 25-fold better than the T2-R strain. This result demonstrates that the high transformation efficiency displayed by the T2 strain is to a large extent due to the nonsense mutation that disrupts the function of its *clpC* gene. Compared with the WT Δ*clpC* mutant, the transformation rate of the T2-R strain is slightly higher when the strains are grown in THG and induced to competence by the STP pheromone. In the absence of the STP pheromone, i.e. in uninduced cultures, no kanamycin-resistant mutants were ever obtained with the WT Δ*clpC* mutant. In contrast, a low number of transformants was consistently obtained with the T2-R strain (Table 2). Consequently, it appears that the T2 strain has acquired mutations in addition to the $G_{1630} \rightarrow T_{1630}$ nonsense mutation that contribute to its enhanced transformability compared with the Δ*clpC* mutant.

ClpC affects ComX accumulation in STP-induced cells

To investigate whether ClpC mediates proteolytic degradation of ComX we exchanged the *comX* gene in the pXL plasmid with a His-tagged version. The 6× His tag was inserted at the C-terminal end of ComX. The resulting pXL-His plasmid was introduced into the WT and Δ*clpC* strains by electroporation giving rise to the WT-His and Δ*clpC*-His strains, respectively. Since the Δ*clpC* strain originally contained the pXL plasmid, it had to be cured of this plasmid before introduction of pXL-His. To compare the levels of ComX in the WT-His and Δ*clpC*-His strains, samples from STP-induced cultures were collected at different time points and analysed by Western blotting. Overnight cultures were diluted in fresh prewarmed THG and cultivated at 37 °C until they reached OD_{492} 0.05. At this optical density 250 ng STP ml^{-1} was added to induce ectopic expression of the ComX-His protein. After further incubation at 37 °C, samples for Western analysis were withdrawn from WT-His and Δ*clpC*-His cultures when they reached OD_{492} 0.2, 0.4 and 0.6. The results showed that ClpC mediates proteolysis of ComX during the time period immediately following STP induction, i.e. until the cultures reached OD_{492} 0.2. However, 1.5 generations later the picture was radically changed. In samples collected from cultures at OD_{492} 0.6, ComX had accumulated to high levels in WT cells, while its concentration in Δ*clpC* cells was strongly reduced (Fig. 3).

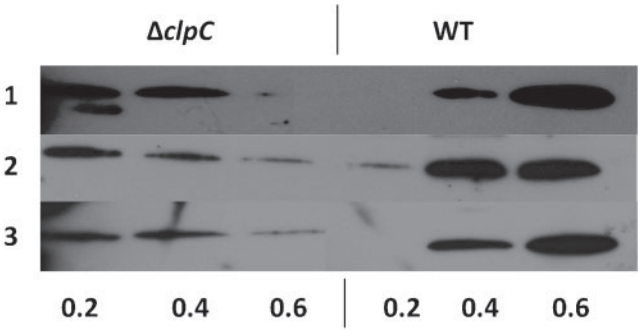

Fig. 3. Western blot analyses comparing levels of ComX-His expression in *S. thermophilus* wild-type (WT) and Δ*clpC* strains grown at 37 °C in THG medium. Both strains carry the pXL-His plasmid. ComX-His expression was induced by addition of STP (250 ng ml^{-1}) at OD_{492} 0.05. Samples for Western analysis were collected when cultures reached OD_{492} 0.2, 0.4 and 0.6. The results of three independent experiments are shown.

DISCUSSION

Our results show that under some conditions, ClpC mutants display increased expression of late competence genes and higher transformation efficiency than the corresponding WT strain. The effect of ClpC is best seen when the level of ComX expression is low, such as in the uninduced Δ*clpC* mutant carrying the pXL plasmid (Fig. 1a and Table 2). In this strain, the leaky bacteriocin promoter controlling transcription of *comX* allows production of small amounts of ComX in the absence of the STP inducer. Luciferase reporter assays carried out with uninduced Δ*clpC* and WT cells, both containing the pXL plasmid, revealed a huge difference in late gene expression (Fig. 1a). Since deletion of the *clpC* gene does not stimulate the rate of *comX* transcription (Fig. 1d), we concluded that the high *luc* activity exhibited by the uninduced Δ*clpC* strain is most likely due to increased stability of the ComX protein.

To obtain additional evidence for this hypothesis we constructed the plasmid pXL-His, which encodes a His-tagged version of ComX driven by an STP-inducible bacteriocin promoter. When WT and Δ*clpC* bacteria carrying the pXL-His plasmid reached OD_{492} 0.05, STP was added to induce expression of ComX. Samples were collected at OD_{492} 0.2, 0.4 and 0.6 and subjected to Western analysis. Interestingly, ComX-His was undetectable in two of three independent Western analyses carried out with WT bacteria harvested at OD_{492} 0.2, i.e. two generations after the STP pheromone was added (Fig. 3). In the third experiment, ComX-His was detected, but the band was very weak. In contrast, strong bands representing ComX-His were obtained with the Δ*clpC* mutant (Fig. 3). These data show that ClpC mediates degradation of ComX at low cell densities in the period immediately following STP induction. This is in agreement with the results of the reporter assay experiments presented in Fig. 1(a). Induction of ComX expression in these experiments was performed exactly as in the Western analyses, i.e. by addition of STP at OD_{492} 0.05. When the experiment was carried out like this, ComX in a Δ*clpC* mutant is not degraded by the ClpCP complex during a period corresponding to about two generations post-induction (Fig. 3). As a consequence, the concentration of ComX required to

elicit strong transcriptional activation of the late competence genes is reached in both induced and uninduced cells, even though uninduced cells produce much less ComX than the corresponding STP-treated cells. The results displayed in Fig. 1(a) also show that light emission peaks earlier in Δ*clpC* than WT cultures. The reason for this is that ComX is not degraded in Δ*clpC* cultures in the period following STP induction and therefore accumulates and triggers late gene expression more rapidly.

ClpC-mediated degradation of ComX vanished at higher ODs, i.e. about three generations after STP induction. The results depicted in Fig. 3 clearly show that the level of ComX-His increases with time and cell density in WT cells, while the Δ*clpC* mutant cells behave in the opposite manner displaying very low ComX levels at OD_{492} 0.6. The increasing amounts of ComX-His observed in WT cells after STP induction presumably results from the combined effects of continuous high expression of ComX-His and saturation of ClpC. However, the existence of additional mechanisms controlling the levels and activity of this sigma factor cannot be ruled out. The virtual disappearance of ComX-His in Δ*clpC* cells at OD_{492} 0.6 was totally unexpected. In theory, it might be caused by ComX-His degradation, shutdown of ComX-His synthesis, or a combination of the two. The STP peptide pheromone used to induce ComX synthesis, concomitantly induces transcription of genes involved in bacteriocin production in *S. thermophilus*. It is therefore conceivable that the increase in bacteriocin-related protein synthesis resulting from the use of STP to some degree affects the kinetics of ComX degradation by the Clp proteolytic complexes and that the effect is not the same in WT and Δ*clpC* cells. More research is required to elucidate these matters.

Previous studies have shown that viable deletion mutants of *clpC*, *clpE*, *clpL* and *clpP* can be generated in *S. pneumoniae*. Only one Clp protein, ClpX, was found to be essential in the pneumococcus (Robertson *et al.*, 2003; Piotrowski *et al.*, 2009b). As we were unsuccessful in generating a *clpX* mutant in *S. thermophilus*, it is reasonable to assume that ClpX is essential also in this species. In *S. pneumoniae* it was demonstrated that the requirement for *clpX* is relieved by deletion of *spr1630*, a gene of unknown function (Piotrowski *et al.*, 2009b). A corresponding mechanism cannot account for our results, however, since the *spr1630* gene is missing from the genome of *S. thermophilus* LMG 18311.

Characterization of pneumococcal strains with non-functional *clpP* genes revealed that these mutants displayed increased expression of early and late competence genes, most likely because Clp proteases inhibit expression of the *comCDE* operon under conditions inappropriate for competence development (Chastanet *et al.*, 2001; Robertson *et al.*, 2002). A similar observation has been made for *Streptococcus pyogenes* (Opdyke *et al.*, 2003). Recently, deletion of the chaperone members of the Clp family demonstrated that ClpE affects the stability of ComX in *S. pneumoniae*. Piotrowski *et al.* (2009a) found that the ClpEP complex contributes to the degradation of the ComX protein in the period following competence induction. The competent state only lasts for about 40 min, after which it is shut down by an unknown mechanism (Håvarstein *et al.*, 1995). It has therefore been speculated that ClpEP degradation of ComX might be responsible for the cessation of competence in *S. pneumoniae*. This turned out not to be the case, however, as transcription of the late genes and consequently the competent state, terminates despite persistently high levels of ComX in a Δ*clpP* mutant (Piotrowski *et al.*, 2009a). Interestingly, our results show that deletion of the *clpE* gene in *S. thermophilus* causes significantly increased late gene expression (Fig. 1b). It is therefore possible that ClpE serves similar functions in *S. thermophilus* and *S. pneumoniae* with respect to competence regulation.

The Clp ATPase, ClpL, which appears to be present only in Gram-positive bacteria, is involved in tolerance to temperature stress in *S. thermophilus*. Varcamonti *et al.* (2006) showed that expression of the ClpL protein increases in cells subjected to heat and cold shock. *S. thermophilus* cells lacking a functional *clpL* gene grew significantly slower at 20 °C and were much less able to tolerate exposure to 60 °C. The observed thermosensitivity of the *clpL* mutant suggests that ClpL functions as a chaperone that assists in protein folding. ClpL lacks the tripeptide ClpP recognition sequence (IGF loop) and is therefore not able to form a proteolytic complex with ClpP (Frees *et al.*, 2007). Consequently, ClpL is presumably not involved in controlling the stability and activity of transcriptional regulators. Our finding that disruption of the *clpL* gene does not affect expression of the late competence genes in STP-induced cells (Fig. 1b) is in accordance with the view that ClpL mediates protein folding rather than protein degradation.

In contrast with *S. pneumoniae*, where inactivation of *clpC* does not significantly affect expression of early and late competence genes (Chastanet *et al.*, 2001), ClpC clearly has a strong effect on the stability of the ComX protein in *S. thermophilus*. Together our results indicate that the biological role of ClpC is to suppress expression of the late competence genes under conditions that are suboptimal for competence development. If left unchecked, it is likely that low-level activation or just transcriptional noise could cause gradual accumulation of intracellular ComX, leading to development of the competent state at an inappropriate time and place. Apparently, ClpC prevents this from happening by degrading ComX. However, under conditions that strongly favour competence development in *S. thermophilus*, ClpC is presumably quickly saturated by high levels of ComX and is therefore not able to block late gene expression and development of the competent state.

The competence-up mutant, termed T2, turned out to be a natural Δ*clpC* mutant. Our results show that the T2 strain transforms considerably better than the *S. thermophilus* LMG 18311 wild-type strain under most conditions tested

(Table 2). Considering that deletion of the *clpC* gene has a strong positive effect on *luc* expression in uninduced WT cells carrying the pXL plasmid (Fig. 1a), it is reasonable to assume that the T2 phenotype results from a non-functional *clpC* gene. To further investigate this, the $G_{1630} \rightarrow T_{1630}$ nonsense mutation was back-mutated to the wild-type sequence, giving rise to the T2-R strain. The transformation efficiency of T2-R strain dropped 25-fold compared with the T2 strain (Table 2). Similarly, the *luc* expression in uninduced cells dropped about fivefold compared with the T2 strain (Fig. 2a, d). These results confirm that the T2 phenotype is predominantly the result of the $G_{1630} \rightarrow T_{1630}$ nonsense mutation. However, there might be additional changes in the T2 genome that contribute to its elevated transformation rate. This is most clearly indicated in the luciferase reporter assays presented in Figs 1(a) and 2(d). Light emission is at least fivefold higher in the uninduced T2-R strain compared with the uninduced WT strain, demonstrating that low levels of ComX activate late gene transcription more efficiently in T2-R than in WT cells. With respect to transformation efficiency, the difference between the T2-R and WT strains is relatively small. In THG medium, STP-induced T2-R cells transform only slightly better than WT cells treated the same way. However, a clear difference was observed between uninduced cultures of the two strains, i.e. at conditions where the intracellular concentration of ComX is low. A few transformants were consistently obtained with the T2-R strain, but no transformants were ever observed when Kan^R-DNA was added to the WT strain (Table 2). In sum, our data show that although the T2 phenotype is largely caused by the $G_{1630} \rightarrow T_{1630}$ nonsense mutation, the T2 strain probably contains additional mutations that further increase its transformability.

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Paper II

Deletion of the murein hydrolase CbpD reduces transformation efficiency in *Streptococcus thermophilus*

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Running title: CbpD affects transformability in *S. thermophilus*

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Summary

Recently it has been shown that *Streptococcus thermophilus* is competent for natural genetic transformation. This property is widespread among streptococci and might include all members of the genus. Upon entering the competent state streptococci start transcribing a number of competence-specific genes whose products are required for binding, uptake and processing of transforming DNA. In addition to the core competence genes, competent streptococci express a number of accessory genes that are dispensable for transformation in the laboratory, but presumably play an important role under natural conditions. In *Streptococcus pneumoniae* one of these accessory genes encodes a competence-specific murein hydrolase termed CbpD. Experimental evidence indicates that pneumococcal CbpD is part of a predatory mechanism that lyse non-competent sister cells or members of closely related species in order to release homologous DNA that can be taken up by the competent attacker cells. Competent *S. thermophilus* LMG18311 cells produce a CbpD-like protein, Stu0039, which might have the same or a similar function. In the present study we have characterized this protein and showed that it is a murein hydrolase with a novel type of cell surface binding domain. Furthermore, we show that Stu0039 is rapidly inactivated by H_2O_2 produced during aerobic growth of *S. thermophilus.* We propose that this inactivation mechanism has evolved for self-protection purposes to prevent extensive autolysis in a competent population. Interestingly, in contrast to pneumococcal CbpD, which does not affect the transformation properties of the producer strain, deletion of Stu0039 reduces the transformability of *S. thermophilus*.

Introduction

In *Streptococcus pneumoniae*, the best studied naturally transformable member of the genus *Streptococcus*, it has been shown that a secreted murein hydrolase termed CbpD (hereafter called CbpD-Sp) is part of the competence regulon (Peterson *et al.,* 2004; Guiral *et al*., 2005; Kausmally *et al.,* 2005). Through the action of CbpD-Sp, competent pneumococci are able to kill and lyse their non-competent siblings and bacteria belonging to closely related species such as *Streptococcus mitis* and *Streptococcus oralis* (Johnsborg *et al*., 2008). To protect themselves competent pneumococci produce ComM, an integral membrane protein that neutralizes the action of CbpD-Sp by an unknown mechanism (Håvarstein *et al.*, 2006). CbpD-Sp consists of three different types of domains: an N-terminal CHAP domain, two central SH3b domains and a C-terminal choline-binding domain consisting of four choline-binding repeats. The CHAP domain 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 (Bateman & Rawlings, 2003; Rigden *et al.*, 2003; Layec et al., 2008). The choline-binding domain attaches CbpD-Sp non-covalently to choline moieties decorating the wall- and lipoteichoic acids of *S. pneumoniae* and related streptococci (Sánches-Puelles *et al.*, 1990), while evidence indicates that the SH3b domains bind to the peptidoglycan part of the cell wall (Eldholm *et al.*, 2010).

Studies have shown that transfer of antibiotic resistance markers from non-competent to competent pneumococci in mixed cultures is much more efficient when the competent cells have a functional *cbpD-Sp* gene (Johnsborg *et al*., 2008). The same holds true for *in vitro* transfer of antibiotic resistance genes from *S. mitis* and *S. oralis* to competent *S. pneumoniae* cells (Johnsborg *et al*., 2008). Consequently, it has been proposed that CbpD-Sp and ComM together constitute a predatory mechanism directed against related strains and species that has evolved to

increase the ability of competent pneumococci to acquire homologous DNA under natural conditions, i.e. in multispecies biofilms in the nasopharynx (Johnsborg *et al*., 2008; Johnsborg & Håvarstein, 2009).

BLASTP searches in the NCBI database with CbpD-Sp as a query revealed that the genomes of a number of streptococcal species encode a CbpD-like protein (Claverys *et al.*, 2007). In every case these *cbpD* genes contain a so called com-box in their promoter regions, demonstrating that they are part of the competence regulon in their respective species. Whereas all of these CbpD-like proteins possess a highly conserved N-terminal CHAP domain, their C-terminal regions might be totally unrelated. This probably reflects that the C-terminal regions, which are thought to attach the catalytic CHAP domain to its substrate, have different binding specificities. *S. thermophilus* encodes a CbpD-like protein (hereafter called CbpD-St) that has a C-terminal region with no known function. BLASP searches showed that related regions or domains are only found in CbpD-like proteins from *Streptococcus salivarius*, *Streptococcus vestabularis*, *Streptococcus pyogenes*, *Streptococcus equi*, *Streptococcus uberis* and some other streptococcal species.

Several streptococcal species do not encode a competence-specific murein hydrolase of the CbpD-type. Instead they express an essentially unrelated murein hydrolase termed LytF during competence. Very recently we have shown that the LytF protein of *Streptococcus gordonii* contributes to increased gene exchange between different *S. gordonii* strains in mixed cultures by a similar predatory mechanism as described for *S. pneumoniae* (unpublished results, Berg, K.H., Ohnstad, H. & Håvarstein, L.S.) Thus, LytF appears to be a functional analogue of CbpD-Sp.

In the present work we show that CbpD-St from *S. thermophilus* has muralytic activity that causes lysis of a subfraction of the cells in a competent culture. Furthermore, we demonstrate that its C-terminal region targets CbpD-St to the equatorial region of the *S. thermophilus* cell surface, and that its N-terminal catalytic CHAP domain is easily inactivated by oxidizing agents. Unexpectedly, in contrast to CbpD-Sp and LytF, which do not affect the transformability of their hosts, our results indicate that *S. thermophilus* LMG18311 mutants lacking CbpD-St have reduced transformability compared to their parental strain.

Methods

Bacterial strains and growth media. Bacterial strains and plasmids used in this work are described in Table 1. Todd-Hewitt broth supplemented with 0.8% glucose (THG) was used to grow strains of *S. thermophilus* LMG 18311. HJGL agar plates were used for selection of *S. thermophilus* transformants in experiments carried out to estimate transformation efficiency. HJGL agar plates were prepared by adding 1.5% (w/v) agar to HJGL medium. HJGL medium consists of Hogg-Jago glucose broth (3% tryptone, 1% yeast extract, 0.2% beef extract, 0.5% KH_2PO_4, and 0.5% glucose) supplemented with 0.5% lactose. *Escherichia coli* was grown in solid or liquid Luria-Bertani (LB) medium as described by Sambrook *et al*. (1989). All incubations were carried out at 37 °C, and all optical density measurements of bacterial cultures were performed spectrophotometrically at 492/550/600 nm. When antibiotic selection was required the following concentrations were used: erythromycin, 500 μg ml^{-1} for *E. coli* and 2 μg ml^{-1} for *S. thermophilus*; streptomycin, 100 μg ml^{-1}; spectinomycin, 150 μg ml^{-1}; kanamycin, 100 μg ml^{-1}; ampicillin, 50 μg ml^{-1}; rifampicin, 2 μg ml^{-1}.

Construction of *cbpD-St* deletion mutants. The *cbpD-St* gene (*stu0039*) of *S. thermophilus* LMG 18311 was disrupted by replacing the native gene with an insertion cassette consisting of a kanamycin resistance gene flanked by two ~1,000-bp DNA fragments corresponding to the 5' and 3' regions of the *cbpD-St* gene. Two different *ΔcbpD-St* mutant strains, ST0039-1 and ST0039-2, were constructed. In the ST0039-2 strain the kanamycin resistance gene was inserted in the same direction as the native *cbpD-St* gene, while it was the other way around in ST0039-1. To make ST0039-1, the kanamycin resistance gene was amplified from the pEW13 vector (Podbielski *et al.,* 1996), using the Kan-F and Kan-R primers (Table 2). Next, the 5' and 3' flanking fragments of the *cbpD-St* gene were amplified in two separate PCR reactions using the primer pairs Up-0039-F / Up-0039-R1 and Down-0039-F1 / Down-0039-R (Table 2) and genomic DNA from *S. thermophilus* LMG 18311 as template. In the final step, all three fragments were stepwise joined by overlap-extension PCR by using the appropriate outer primers. The final product, the gene disruption cassette, was purified with a PCR purification kit obtained from Macherey-Nagel and cloned into the pCR 2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions. pCR 2.1-TOPO containing the disruption cassette was purified from liquid culture using MN Midi and used directly to transform *S. thermophilus* LMG 18311 by natural transformation. The ST0039-2 strain was made in the same way except that the primers Up-0039-R1 and Down-0039-F1 were exchanged with the primers Up-0039-R2 and Down-0039-F2, respectively.

Construction of the p0039 plasmid used for complementation studies. Construction of the p0039 plasmid was performed by amplifying the native *cbpD-St* gene from *S. thermophilus* LMG 18311, including the promoter region, using the primers CbpD-comp-F and CbpD-comp-R

(Table 2). The resulting PCR fragment was purified with a PCR purification kit obtained from Macherey-Nagel, cleaved with *Xho*I and *Pst*I and ligated into corresponding sites in precleaved pTRKH2, creating the p0039 plasmid. All PCR reactions were carried out with the Phusion high-fidelity DNA polymerase (Finnzymes), and the final construct was checked by complete sequencing. The p0039 plasmid was electroporated into electrocompetent ST0039-2 cells as described previously (Biørnstad & Håvarstein, 2011). Transformants were selected on THG agar plates containing 2 μg ml^{-1} of erythromycin.

Natural transformation of *S. thermophilus* LMG 18311 and mutants derived from it. *S. thermophilus* strains were grown overnight at 37°C in THG. The following day cultures were diluted in prewarmed medium to OD_{550} = 0.05. Then, 1-ml samples were transferred to microcentrifuge tubes, supplemented with 900 ng ml^{-1} of the competence pheromone ComS* (NH_2-LPYFAGCL-COOH), and further incubated at 37°C. When OD_{550} reached 0.3, transforming DNA was added, and incubation was continued for 2 hours at 37°C. Samples were then put on ice, serially diluted, and spread on HJGL agar plates containing the appropriate antibiotic. When comparing the transformability of the *ΔcbpD-St* mutants to their parental strain (Table 3), 3 μg ml^{-1} of p0055 plasmid or 2 μg ml^{-1} of genomic DNA were used per transformation experiment.

Zymogram analysis. Zymogram analysis, performed according to the description by Leclerc and Asselin (1989), was used to detect muralytic activity in whole cell extract from competent and noncompetent cultures of *S. thermophilus* LMG 18311 (Wt), ST0039-2 and ST0039-C. Overnight cultures of these strains grown at 37°C in THG supplemented with the appropriate

antibiotic, were diluted to OD_{550} = 0.05 in the same medium. Following dilution, the cultures were immediately induced to competence by addition of ComS* (900 ng ml^{-1}) and further incubated at 37°C until reaching OD_{550} = 0.4. Samples (10 ml) were then withdrawn, and the bacteria were pelleted by centrifugation. Pellets were resuspended in 200 µl Laemmli (1970) sample buffer and stored at - 80°C. After heating to 95°C for 5 min, 15 µl samples were separated by SDS-PAGE using a 4% stacking gel and a 10% resolving gel. As substrate for potential murein hydrolases, heat killed bacteria were mixed into the resolving gel solution before it had polymerized. Substrate cells were prepared by growing a 300 ml culture of *S. thermophilus* LMG 18311 to OD_{550} = 0.2. Then the cells were pelleted by centrifugation and washed once in 5 ml ice cold Tris-HCl (20 mM, pH 7.4) buffer containing 100 mM NaCl. After washing followed by centrifugation, the resulting pellet was dissolved in 1.25 ml Tris-HCl (1.5 M Tris-HCl, pH 8.8) and heat treated for 10 min at 95°C. This 1.25 ml mixture of Tris-HCl and cells was subsequently used to prepare the resolving gel instead of a corresponding volume of pure Tris-HCl buffer. Electrophoresis was performed at 2.5 V cm^{-1}, after which gels were washed in distilled deionized water (2 x 30 min) and incubated in refolding buffer (50 mM NaCl, 20 mM $MgCl_2$, 0.5% Triton X-100 and 20 mM Tris-HCL, pH 7.4). Gels were incubated in refolding buffer until lytic activity was observed as bands of clear zones in the opaque gel.

Quantification of β-galactosidase release. Overnight cultures grown at 37°C were diluted to OD_{600} = 0.05 in freshly prepared, prewarmed THG. Following dilution, the cultures (10 ml) were induced to competence by addition of 900 ng ml^{-1} ComS*, and further incubated in a water bath at 37°C. Uninduced samples, receiving no peptide pheromone, were run in parallel as negative controls. After 1 hour, 1.5 ml samples were withdrawn and filtrated (0.2-µm filter) to obtain cell-

free supernatants. β-galactosidase assays of supernatants were carried out in microcentrifuge tubes at 30°C and contained 240 µl of 5× Z buffer [5 mM $MgCl_2$, 250 mM β-mercaptoethanol, 50 mM KCl, 0.3 M $Na_2HPO_4 \cdot 7H_2O$, 0.2 M $NaH_2PO_4 \cdot H_2O$, 4 mg ml^{-1} *o*-nitrophenyl-β-D-galactopyranoside (ONPG), pH 7.0], 800 µl of supernatant, and 160 µl of THG medium. After 20 min at 30°C reactions were terminated by adding 0.05 ml of a 1 M Na_2CO_3 solution and samples were placed on ice. Hydrolysis of ONPG was recorded in a spectrophotometer at 420 nm. β-galactosidase activity was calculated according to Miller (Miller, 1972).

To measure total β-galactosidase activity (intracellular + extracellular), 1.5 ml samples collected in parallel with the samples assayed only for extracellular β-galactosidase activity, were mixed with 0.5 g of acid-washed glass beads (≥106 microns). Then, the bacterial cells were disrupted using a FastPrep-24 cell homogenizer as described by the manufacturer (MP Biomedicals). Following disruption of bacterial cells, glass beads and cell debris were removed by centrifugation at 13 000 rpm for 1 min. β-galactosidase assays of cells were carried out as described above using 240 µl of 5× Z buffer, 200 µl cleared lysate, and 760 µl of THG medium.

Construction of GFP-CbpD-St fusion protein.

The gene ecoding EmGFP, including the upstream vector-encoded 6 x His-tag, were amplified by PCR using the primers 202-GFP-F and 230-GFP-R with the pRSET-EmGFP plasmid (Invitrogen) as template. Next, the C-terminal domain of CbpD-St was amplified using the primers CbpD-St-F and CbpD-St-R with genomic DNA from *S. thermophilus* LMG18311 as template. Then, the two fragments were fused by overlap extension PCR using the outer primers 202-GFP-F and Cbp-St-R. Unique restriction sites were introduced at the 5'-ends of the 202-GFP-F (*Nhe*I) and Cbp-St-R (*Xho*I) primers during synthesis. The fragment generated by overlap

extension PCR was digested with *Nhe*I and *Xho*I, and ligated into the corresponding restriction sites in the pRSET A expression vector (Invitrogen). The resulting recombinant plasmid (pHOG1) was transformed into BL21(DE3)pLysS cells (Novagen) for expression of the fusion protein. Briefly, after reaching OD_{600} = 0.5, a culture of BL21(DE3)pLysS cells harboring the pHOG1 plasmid was subjected to 1 mM IPTG and incubated at 30ºC with shaking (200 rpm) for 2 hours. The cells were lysed by repeated freezing and thawing using liquid nitrogen and a 42ºC water bath. After removing the cell debris by centrifugation (70 000 x g for 10 min), the supernatant was filtered through a 0.45 µm filter. His-tagged GFP-CbpD-St was purified from the filtered supernatant by the use of Protino Ni-TED columns (Macherey Nagel) as described by the manufacturer. Fluorescence microscopy was performed as described by Eldholm *et al.* (2010).

Results

Deletion of the *cbpD-St* gene reduces transformation efficiency in *S. thermophilus* LMG18311

We have previously shown that deletion of the *cbpD-Sp* and *lytF* genes from *S. pneumoniae* and *S. gordonii*, respectively, does not alter the transformation properties of the mutants compared to their parental strains (Johnsborg *et al*., 2008; unpublished results, Berg, K.H., Ohnstad, H. & Håvarstein, L.S.). Consequently, when starting the present study on CbpD-St from *S. thermophilus* we expected that a *cbpD-St* mutant and the corresponding wild-type strain would be equally transformable when subjected to exogenously added DNA. As donor-DNA we used saturating amounts of the plasmid p0055, which consists of the pCR2.1-TOPO vector carrying a spectinomycin resistance gene (Spc^R) flanked by ~900 bp fragments corresponding to the

upstream and downstream regions of the *stu0055* gene. The *stu0055* gene encodes a non-essential 74-amino acid protein of unknown function. To our surprise, the transformation efficiency of the *cbpD-St* deficient strain ST0039-1 was about eighteen-fold lower than that of its LMG18311 parental strain (Table 3). In the ST0039-1 *cbpD-St* knockout mutant the kanamycin resistance gene is inserted in the opposite direction of the native *cbpD-St* gene. We therefore speculated that transcriptional readthrough from the kan^R promoter into the downstream *de novo* purine biosynthesis genes *purD, purE and purK* might have caused the observed reduction in transformability of the ST0039-1 mutant strain. Hence, we constructed a *cbpD-St* knockout mutant, ST0039-2, with the kan^R gene oriented in the opposite direction. Analysis of the transformability of this mutant strain showed that it is reduced about seventeen-fold compared to wild-type (Table 3), i.e. the same result as obtained with the ST0039-1 strain.

To ascertain that the reduced transformability observed with the ST0039-1 and ST0039-2 strains is caused by the loss of CbpD-St, we sought to complement the *ΔcbpD-St* mutation by transforming the p0039 vector carrying the *cbpD-St* gene and its promoter into the ST0039-2 strain. Assessment of the transformation efficiency of the resulting ST0039-C strain showed that ectopically expressed CbpD-St was able to complement the loss of the endogenous *cbpD-St* gene (Table 3). Together these results strongly indicate that the absence of CbpD-St during competence decreases the transformability of *S. thermophilus* LMG18311.

To make sure that the effect of CbpD-St on transformability does not depend on the nature of the donor DNA, the experiment was repeated with genomic Rif^R-DNA purified from the RH-ST25 strain. As would be expected, the transformation efficiency was significantly lower with genomic Rif^R-DNA than with p0055 plasmid DNA (Table 3). When genomic DNA is used, a much smaller fraction of the DNA taken up and incorporated into the genomes of competent

bacteria contains the selectable marker gene. Hence, fewer antibiotic resistant transformants are obtained. In this case the CbpD-St deficient strain displayed a 6.5-fold reduction in transformation efficiency compared to its parental strain (Table 3).

CbpD-St from *S. thermophilus* has murein hydrolase activity

The transformation experiments described above show that CbpD-St from *S. thermophilus*, in contrast to pneumococcal CbpD-Sp, has an impact on transformability. The two CbpDs possess highly homologous N-terminal CHAP domains, but their C-terminal domains are completely unrelated. As mentioned in the introduction section, the CHAP domain of CbpD-Sp 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. To determine whether CbpD-St functions as a murein hydrolase we performed zymogram analyses with total cell extracts from competent and noncompetent cells of *S. thermophilus* LMG18311 (WT) and its CbpD deficient derivative ST0039-2. To detect muralytic activity, *S. thermophilus* LMG18311 cells were incorporated in the SDS-PAGE resolving gel as substrate. A clearing zone corresponding to a protein of about 30 kD appeared in the lane loaded with cell extract from competent wild-type cells (Fig. 1, lane 3). This clearing zone was not present in lane 2, which contained total cell extract from noncompetent wild-type cells. Furthermore, no clearing zone corresponding to the predicted size of CbpD-St (~27.2 kD) was detected in lane 5, which had been loaded with a sample prepared from competent cells of the CbpD deficient strain ST0039-2. These findings clearly show that CbpD-St functions as a murein hydrolase. As expected, a clearing zone was detected in the lane that had been loaded with a sample prepared from competence-induced ST0039-C cells (Fig. 1,

lane 7), demonstrating that the restored transformation efficiency of this strain is due to the presence of CbpD-St.

CbpD-St binds to the equatorial region of *S. thermophilus* cells

CbpD-Sp and CbpD-St has totally unrelated C-terminal regions. In CbpD-Sp this region consists of two SH3-domains and a choline-binding domain whose combined functions are to attach CbpD-Sp to the surface of target cells. In the case of CbpD-St the domain corresponding to the C-terminal region shows no close homology to proteins or domains with a known function. However, since CbpD-St is a murein hydrolase it is reasonable to assume that its C-terminal region functions as a cell wall-targeting domain. To investigate these matters we constructed a fusion protein in which the CHAP domain of CbpD-St was replaced with green fluorescence protein (GFP). To simplify purification of the fusion protein, a His-tag was added to the N-terminus of the GFP-domain. Fluorescence microscopy revealed that the fusion protein binds strongly to the *S. thermophilus* cell surface, and that it specifically localizes to the equatorial region (Fig. 2). In a previous study we showed that a pneumococcal GFP-CbpD fusion protein constructed in the same manner, bound to the cell equator as well as the septal and polar regions (Eldholm *et al*., 2010). In contrast, binding of the GFP-CbpD-St construct to the poles and septal regions of *S. thermophilus* LMG18311 cells is either very weak or absent.

Dithiothreitol (DTT) and catalase boost the effect of CbpD-St

As stated above, the effect of CbpD-St on the transformability of *S. thermophilus* LMG18311 was totally unexpected as the previously characterized fratricins CbpD-Sp and LytF display no such effect. When expressed during competence CbpD-Sp and LytF are able to lyse susceptible

cells, causing the release of DNA and other intracellular contents into the extracellular milieu. To determine whether CbpD-St is able to lyse *S. thermophilus* LMG18311 cells we measured the amount of intra- and extracellular β-galactosidase in competent and noncompetent cultures of this bacterium. The β-galactosidase produced by *S. thermophilus* is an intracellular protein. The level of β-galactosidase activity in the growth medium relative to the total amount of β-galactosidase in the culture (intracellular + extracellular) can therefore be used to calculate the percentage of lysed cells. Comparison of competent and noncompetent cultures of *S. thermophilus* wild-type cells showed that the fraction of lysed cells increased from about 0.7% in noncompetent to 4.7% in competence-induced cultures (Table 4). No lysis was detected in competent and noncompetent cultures of the ST0039-2 strain, demonstrating that the observed lysis (~ 4%) in competent wild-type cultures is caused by CbpD-St (Table 4). These results also show that most cells in a competent *S. thermophilus* culture are resistant to CbpD-St under the experimental conditions used. In *S. pneumoniae* and related species such as *S. mitis* and *S. oralis*, competent cells are protected against CbpD by the immunity protein ComM. The genome of *S. thermophilus* does not contain any homologs of the *comM* gene, but it is possible that competent cells of this species are protected by a different immunity mechanism.

We have previously shown that DTT, which is capable of maintaining protein –SH groups completely in the reduced state, stabilizes the activity of pneumococcal CbpD, presumably by protecting the active-site cysteine in its CHAP domain from oxidation (Eldholm *et al.*, 2009). When 1 mM DTT was included in the β-galactosidase assays, cell lysis in the competence-induced wild-type culture increased to more than 30% (Table 4). As before, no lysis was observed in a parallel experiment carried out with the ST0039-2 strain. To directly observe the effect of CbpD-St on the viability of *S. thermophilus* cells growing under standard or

reducing conditions, the experiment shown in Fig. 3 was carried out. In this experiment wild-type and ST0039-2 cultures were diluted to OD_{492} = 0.05 at 37 ºC, and then transferred into a 96-well Corning NBS plate with a clear bottom. The plate was incubated at 37 ºC in an Optima FLUOstar plate reader that measured OD_{492} automatically at 10 min intervals. When appropriate, ComS* and/or DTT was added to the cultures immediately after they were transferred to the 96-well plate. The results depicted in Fig. 3A show that a large fraction of the cell population is killed when the culture is induced to competence in the presence of 1 mM DTT. In the absence of DTT, however, the difference between ComS*-induced and non-induced cultures is small. The results shown in Fig. 3B verify that the large loss of viability observed in competent cultures grown under reducing conditions is due to CbpD-St. These findings strongly indicate that one or more oxidants in the culture medium inactivate CbpD-St shortly after it is translocated across the cytoplasmic membrane.

S. thermophilus is known to produce a Mn-superoxide dismutase (SodA) that converts superoxide anions to O_2 and H_2O_2. We therefore speculated that H_2O_2 might be the (or one of the) oxidizing agent(s) responsible for inactivating CbpD-St. To determine if H_2O_2 contributes to the inactivation of extracellular CbpD-St we added 400 U/ml catalase to the β-galactosidase assay described above instead of DTT. Our result showed that the presence of catalase increased the lytic activity of CbpD-St more than 3-fold compared to competent cultures without catalase. This result demonstrates that H_2O_2 alone, or in combination with other oxidizing agents, rapidly inactivates extracellular CbpD-St under the experimental conditions used.

Discussion

In a very recent study we have shown that most or probably all streptococcal species encode a murein hydrolase that is expressed when the bacteria enter the competent state (unpublished results, Berg, K.H., Ohnstad, H. & Håvarstein, L.S.). These murein hydrolases, collectively called fratricins, come in two major types. So far, BLASTP searches have shown that 20 streptococcal species encode CbpD-like fratricins, while 12 species encode the virtually unrelated LytF-type. CbpD-Sp and CbpD-St both belong to the CbpD-like fratricins, while the fratricin produced by for instance *Streptococcus gordonii* belongs to the LytF-type. Previously we have characterized the properties of CbpD-Sp from *S. pneumoniae* and LytF from *S. gordonii.* In both cases these fratricins have no effect on the transformability of the species producing them, but contribute strongly to increase the efficiency by which DNA is transferred between their respective species *in vitro* (Johnsborg *et al.*, 2008; unpublished results, Berg, K.H., Ohnstad, H. & Håvarstein, L.S.). It has therefore been proposed that the biological function of CbpD-Sp and LytF is to lyse and release DNA from susceptible bacteria in order to provide homologous transforming DNA to the competent recipient cells. The fact that these fratricins have a relative narrow target spectrum supports this view. In the current investigation we discovered that CbpD-St deviates from CbpD-Sp and LytF in one important aspect: it affects the transformability of *S. thermophilus.* Initially, we believed that this result was due to some flaw in the experimental design, but after carrying out relevant control experiments we still saw that CbpD-St proficient strains transformed with higher efficiency than CbpD-St deficient strains. Does this effect of CbpD-St on transformability reflect its true biological function in *S. thermophilus*? Not necessarily. CbpD-St's transformation-enhancing property might just be a side effect of its muralytic activity and/or the experimental conditions used, and does not have to

be evidence of the real function of CbpD-St under natural condition. It is possible, on the other hand, that CbpD-St and the other two fratricins, CbpD-Sp and LytF, serve different biological functions. The *S. thermophilus* LMG18311 strain was originally isolated from yogurt manufactured in the United Kingdom. Sequencing of its genome has shown that the strain is highly adapted to the fermentation of milk products (Bolotin *et al.,* 2004). During adaptation to the dairy environment about 10% of its genes have degenerated into functionless pseudogenes. Interestingly, competence for natural genetic transformation has not been lost in the *S. thermophilus* LMG18311 strain, strongly indicating that this property provides a selective advantage even in the rather narrow and constant environment represented by industrial dairy fermentation (Bolotin *et al*., 2004; Hols *et al*., 2005; Blomqvist *et al.*, 2006; Gardan *et al*., 2009; Fontaine *et al.*, 2010). However, the altered selection pressure experienced by *S. thermophilus* after it was domesticated and no longer subjected to competition from other members of the oral microflora might have changed the properties and biological role of CbpD-St. Investigation of the properties and function of CbpD from the oral commensals *Streptococcus vestibularis* and *Streptococcus salivarius*, the most closely related non-domesticated relatives of *S. thermophilus*, might help answer this question.

How could a murein hydrolase contribute to enhanced transformability? It is conceivable that CbpD-St through its muralytic activity could stimulate DNA uptake by making the cell wall of *S. thermophilus* more permeable to DNA and/or the pseudopili produced during competence. These pseudopili are an essential part of the DNA uptake machinery in competent streptococci (Dubnau, 1999; Peterson *et al*., 2004). Perhaps the cell wall of *S. thermophilus* is more highly cross-linked than the cell walls of *S. pneumoniae* and *S. gordonii.* If so, removal of some of the cross-links by CbpD-St might help facilitate DNA uptake. Presuming that CbpD-St facilitates

DNA uptake in this way, it would be expected that the process takes place where CbpD-St has been shown to bind. This implies that the DNA uptake apparatus of competent *S. thermophilus* cells must be assembled at their equators. Studies conducted on naturally competent *Bacillus subtilis* cells do not support an equatorial location of the DNA uptake apparatus. On the contrary, Hahn *et al.* (2005) have shown that DNA uptake in members of this species takes place at their poles. Although this finding suggests that the cell pole is the site of DNA uptake in competent bacteria, it is of course possible that the cell-surface location of this process varies between species. However, should future studies find that DNA is taken up at the poles of competent *S. thermophilus* cells, it would argue strongly against a facilitator role of CbpD-St in DNA uptake.

In the present study we have shown that aerobically grown *S. thermophilus* generates H_2O_2 that efficiently inactivates CbpD-St produced during competence. Presumably, H_2O_2 oxidizes the active-site cysteine to sulfenic acid, rendering the enzyme non-functional. The finding that CbpD-St rapidly loses its muralytic activity after it has crossed the cytoplasmic membrane is puzzling. The same phenomenon has previously been observed for pneumococcal CbpD-Sp (Eldholm *et al.,* 2009). If spontaneous competence development under natural condition only takes places in the absence of oxygen, the observed oxidative inactivation of CbpD-St by H_2O_2 is just a consequence of the aerobic growth conditions employed in our laboratory experiments and has no biological relevance. However, presuming that spontaneous competence development normally occurs in the presence of oxygen, the observed inactivation might represent a protective mechanism that controls the activity of CbpD-St. In the case of *S. pneumonia,* it has been reported that growth under low oxygen conditions *in vitro* reduces or abolishes spontaneous competence development, indicating that oxygen is required for full competence in this species (Echenique *et al*., 2000). The growth conditions that induce

spontaneous competence development in *S. thermophilus* have not been elucidated, but at least for the LMD-9 strain it has been shown that spontaneous competence development takes place under aerobic conditions (Gardan *et al.,* 2009). Consequently, it is reasonable to assume that the H_2O_2-mediated inactivation of CbpD-St is biologically relevant and functions as a protection mechanism that prevents massive self-lysis in a competent population.

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Table 1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Characteristics	Reference/Source
Strains		
S. thermophilus LMG18311		ATCC (BAA-250)
ST0039-1	*S. thermophilus* LMG18311, but *ΔcbpD-St* by replacement with a kan^R gene. The kan^R-gene was inserted in opposite direction of the native *cbpD-St* gene.	This work
ST0039-2	*S. thermophilus* LMG18311, but *ΔcbpD-St* by replacement with a kan^R gene. The kan^R-gene was inserted in the same direction as the native *cbpD-St* gene.	This work
ST0039-C	ST0039-2 carrying plasmid p0039.	This work
RH-ST25	Rifampicin resistant (Rif^R) *S. thermophilus* LMG 18311 by spontaneous conversion.	This work
Plasmids		
pTRKH2	Shuttle vector carrying an erythromycin resistance gene	O'Sullivan and Klaenhammer, 1993.
p0039	pTRKH2 carrying the *cbpD-St* gene under control of its own promoter.	This work
p0055	pCR2.1-TOPO vector carrying a spc^R gene flanked by the immediate up- and downstream regions of stu0055.	This work
pHOG1	pRSET A (Invitrogen) carrying an insert consisting of EmGFP fused to the C-terminal domain of CbpD-St	This work

Table 2. Primers used in this study

Primer	Primer sequence (5'-3')
Up-0039-F	ATAATGTAGAACTTGTCCCCATTGAC
Up-0039-R1	AGCATGCATATGCATCCGGAGTAAAATG-AAGATGATTTTGAATTTAAGTCT
Up-0039-R2	AAGCTTAAGATCTAGAGCTCGAGGATAA-AATGAAGATGATTTTGAATTTAAGTCT
Down-0039-F1	AAGCTTAAGATCTAGAGCTCGAGGATAA-TTAGACAACTAATTTATCCAAAGGATTT
Down-0039-F2	AGCATGCATATGCATCCGGAGTAATTAG-ACAACTAATTTATCCAAAGGATTT
Down-0039-R	AAAGTGTAAGTCTTGATTATTGTCTTTCAA
CbpD-comp-F	ATTACTCGAGGGACCGCCTCTTAGGAGATT
CbpD-comp-R	AATTACTGCAGACCGTTCTCACGATTGTTCA
Kana-F	ATCCTCGAGCTCTAGATCTTAAGCTT
Kana-R	ACTCCGGATGCATATGCATGCT
202-GFP-F	AGCAATGCTAGCGTGAGCAAGGGCGAGGA
230-GFP-R	CTTGTACAGCTCGTCCATGCCGA
CbpD-St-F	CGGCATGGACGAGCTGTACAAGTCAGGAACTC-AAAATGGAAATCCT
CbpD-St-R	AGCAATCTCGAGTTAAATCTTTTCAAGGACAGA-TTGG

Table 3. Effect of deleting the *cbpD-St* gene on the transformation efficiency of *S. thermophilus*.

S. thermophilus strain	Donor DNA	Transformation efficiency[†]
LMG18311 (WT)	Plasmid p0055	0.27 ± 0.039%
ST0039-1 (*ΔcbpD-St*)	Plasmid p0055	0.015 ± 0.007%
ST0039-2 (*ΔcbpD-St*)	Plasmid p0055	0.016 ± 0.004%
ST0039-C*	Plasmid p0055	0.15 ± 0.05%
LMG18311 (WT)	Genomic Rif^R DNA from strain RH-ST25	0.0013 ± 0.0002%
ST0039-2 (*ΔcbpD-St*)	Genomic Rif^R DNA from strain RH-ST25	0.0002 ± 0.00005%

[†]Transformants (c.f.u.)/total number of bacteria (c.f.u.) x 100 ± SEM. Each transformation experiment was repeated at least three times.

*The ST0039-C strain lacks the native *cbpD-St* gene, but contains a plasmid (p0039) carrying the *cbpD-St* gene under control of its own promoter.

Table 4.

S. thermophilus strain	ComS*	Supplement	Cell lysis (%)*
LMG18311 (WT)	+	None	4.7 ± 1.2
LMG18311 (WT)	–	None	0.7 ± 0.13
ST0039-2 (*ΔcbpD*)	+	None	0
ST0039-2 (*ΔcbpD*)	–	None	0
LMG18311 (WT)	+	DTT	31.6 ± 1.9
LMG18311 (WT)	–	DTT	0.28 ± 0.16
ST0039-2 (*ΔcbpD*)	+	DTT	0
ST0039-2 (*ΔcbpD*)	–	DTT	0
LMG18311 (WT)	+	Catalase	13.8 ± 2.2
LMG18311 (WT)	–	Catalase	0.83 ± 0.38
ST0039-2 (*ΔcbpD*)	+	Catalase	0.28 ± 0.15
ST0039-2 (*ΔcbpD*)	–	Catalase	0.45 ± 0.23

*Miller units in supernatant/ total Miller units (extracellular + intracellular) ± SEM.

Individual experiments were repeated at least three times.

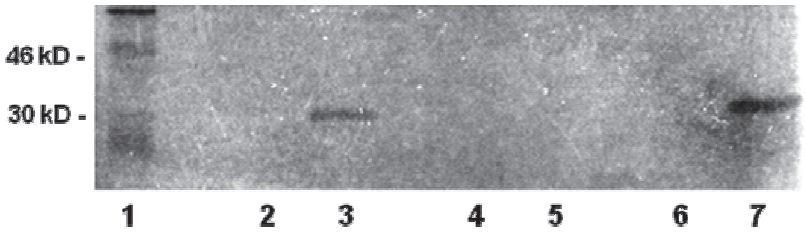

Fig. 1. Murein-hydrolyzing activity in total cell extracts of *S. thermophilus* LMG18311 and derivatives of this strain revealed by zymogram analysis. The dark bands represent clearing zones that result from degradation of *S. thermophilus* LMG18311 cells that have been incorporated in the SDS-PAGE resolving gel. Lane 1: Molecular weight standards, lane 2: Extracts from noncompetent *S. thermophilus* LMG18311cells, lane 3: Extracts from competence induced *S. thermophilus* LMG18311cells, lane 4: Extracts from noncompetent ST0039-2 cells, lane 5: Extracts from competence induced ST0039-2 cells, lane 6: Extracts from noncompetent ST0039-C cells, lane 7: Extracts from competence induced ST0039-C cells.

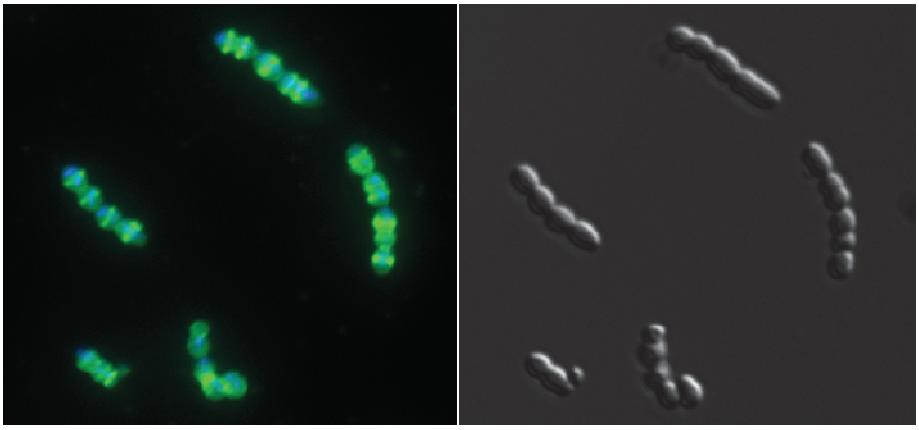

Fig. 2. Binding of a GFP-CbpD-St fusion protein to the surface of *S. thermophilus* LMG18311 cells. The GFP-CbpD-St hybrid protein was made by exchanging the CHAP domain of CbpD-St with green-fluorescence-protein. Left panel: Fluorescence micrograph showing *S. thermophilus* LMG18311 cells stained with the DNA-specific fluorescent probe DAPI (blue) and the GFP-CbpD-St fusion protein (green). Right panel: A differential interference contrast (DIC) image of the cells shown in the left panel.

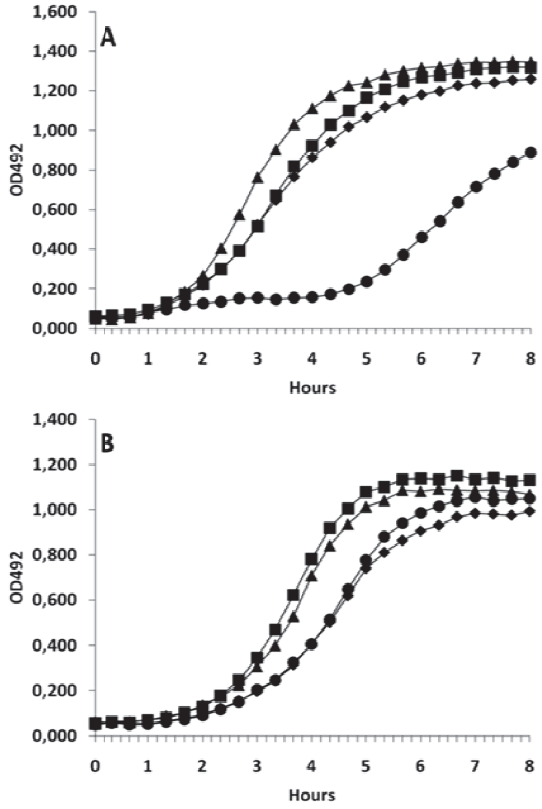

Fig. 3. Effect of dithiothreitol (DTT) on the growth of competent and noncompetent cultures of *S. thermophilus* LMG 18311 and the CbpD-St deficient ST0039-2 strain. In cultures receiving DTT (1 mM) and/or ComS* (900 ng ml^{-1}), these compounds were added at time zero. Panel A: (▲) Uninduced *S. thermophilus* LMG 18311, (■) ComS* induced *S. thermophilus* LMG 18311, (◆) Uninduced *S. thermophilus* LMG 18311 subjected to DTT, and (●) ComS* induced *S. thermophilus* LMG 18311 subjected to DTT. Panel B: (▲) Uninduced ST0039-2, (■) ComS* induced ST0039-2, (◆) Uninduced ST0039-2 subjected to DTT, and (●) ComS* induced ST0039-2 subjected to DTT. These experiments were repeated three times with highly similar results.

Paper III

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

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

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

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

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TABLE 1. Bacterial strains and plasmids

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

TABLE 2. Primers

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

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

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

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

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

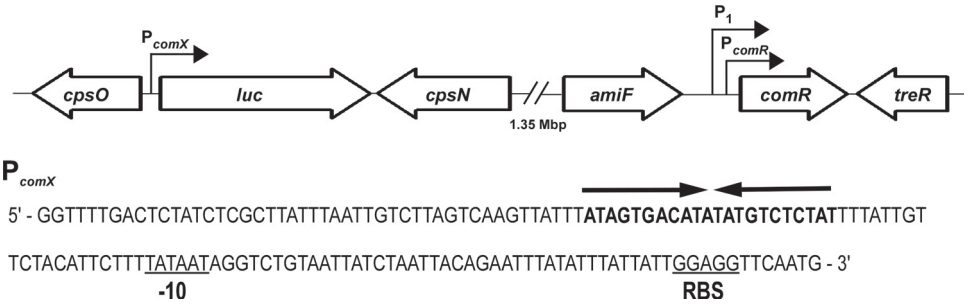

FIG. 1. Schematic diagram depicting two genetic regions of *S. pneumoniae* mutant SPH130 containing the inserted P_{comX}::*luc* and P_1::P_{comR}::*comR* constructs. The promoters P_{comX}, P_{comR}, and P_1 are indicated. The sequence of P_{comX} is shown below the diagram, with the predicted ComR-binding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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

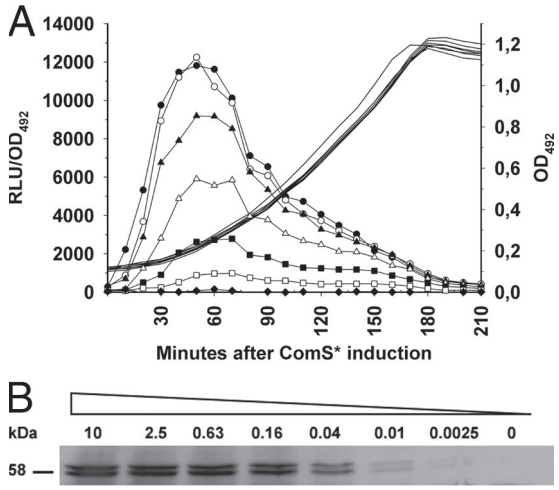

FIG. 2. Bioluminescence (A) and Western analyses (B) showing the level of Luc expression after subjecting SPH130 cells to various concentrations of ComS*. (A) SPH130 cell cultures were induced at an OD_{492} of 0.1 with the following concentrations of ComS*: 0 (◆), 0.08 μM (□), 0.16 μM (■), 0.31 μM (Δ),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

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

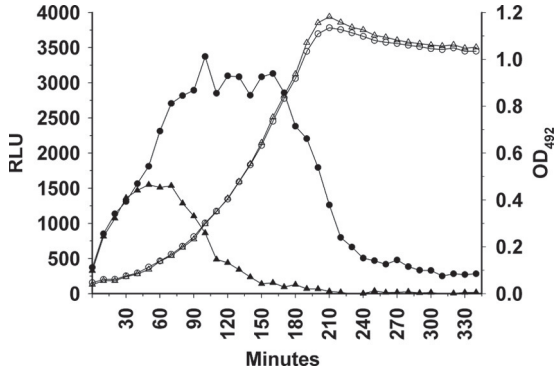

FIG. 3. Decay of luciferase activity over time after removal of ComS* from the growth medium. The SPH130 strain was grown either in the presence of 2 μM ComS* (circles) or in the absence of ComS* (triangles). Bacterial growth is indicated by open symbols, whereas luminescence is indicated by filled symbols.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

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

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

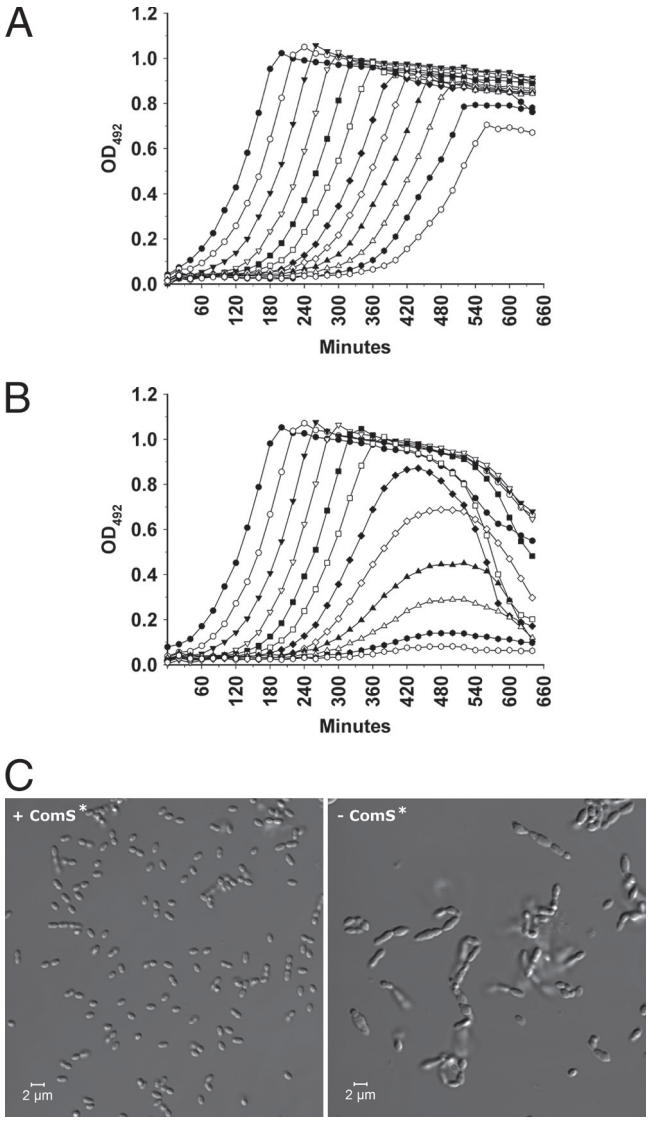

FIG. 4. Effects of *licD1* depletion on growth and morphology of SPH135 cultures. (A) A culture of SPH135 cells grown to an OD_{492} of 0.3 in C medium supplemented with 2 μM ComS* was pelleted, washed once in plain C medium, and resuspended to an OD_{492} of 0.05 in fresh C medium containing 2 μM ComS*.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

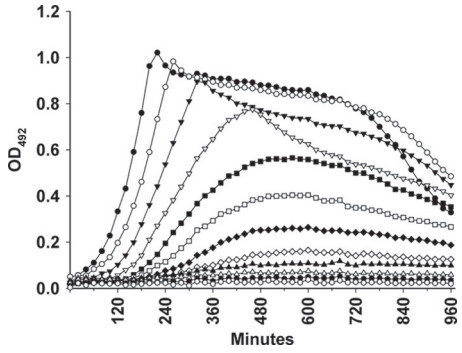

FIG. 5. Depletion of the Kan^r gene makes the SPH136 mutant sensitive to kanamycin. SPH136 cell cultures were grown in C medium containing 2 μM ComS* until they reached an OD_{492} of 0.3. Then, they were pelleted, washed once, and resuspended to an OD_{492} of 0.05 in ComS*-free C medium containing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

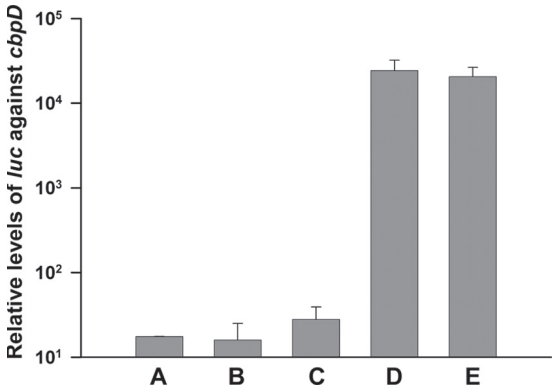

FIG. 6. Expression levels of *luc* relative to the expression levels of *cbpD* measured by real-time RT-PCR. The late competence gene *cbpD* is only expressed in cells that are competent for natural genetic transformation. (A) Background transcription of *luc* was examined in SPH130 cells grown without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

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

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

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

DISCUSSION

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

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

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

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

ACKNOWLEDGMENT

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