EXTRACELLULAR-PEPTIDE CONTROL OF COMPETENCE FOR GENETIC TRANSFORMATION IN *STREPTOCOCCUS PNEUMONIAE*

Jean-Pierre Claverys [1] and Leiv Sigve Havarstein [2]

[1] *Laboratoire de Microbiologie et Génétique Moléculaire, UMR 5100 CNRS-Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, France.* [2] *Department of Chemistry and Biotechnology, Agricultural University of Norway, PO Box 5040, N-1432, As, Norway*

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1. ABSTRACT

Bacteria, which often are subjected to fluctuations in nutrients, temperature, radiation, pH, etc…, adapt to the physico-chemical environment they live in by making the appropriate changes in their gene expression patterns. During the last decades it has become increasingly clear that bacteria, in addition, have a "social life", and that changes in gene expression can also be elicited by the presence of other bacteria. Traditionally bacteria have been viewed as solitary organisms that in general do not interact with other bacteria in a coordinated manner. Recent advances in the field of bacterial cell-to-cell communication has proved this to be a misconception, and mounting evidence now show that bacterial group behaviour is ubiquitous in nature.

Competence for natural genetic transformation in *Streptococcus pneumoniae*, which has been studied for more than seventy years, has become a paradigm for

intercellular communication and cell density dependent regulation of gene expression in Gram-positive bacteria. There has been rapid progress recently in elucidating the molecular mechanisms behind regulation of natural competence in *S. pneumoniae*. In this review, we describe the current status of our knowledge of natural competence in this bacterium, with particular emphasis on the early phase of competence induction.

2. INTRODUCTION

Bacteria communicate with secreted chemical signals to coordinate gene expression within a population, enabling them to act as a group when this is an advantageous strategy for survival (1). To be effective, this group-behaviour depends on population density. Bacteria have therefore evolved a cell-cell communication system termed quorum-sensing that monitors cell density indirectly by measuring the external concentration of specific signaling molecules secreted by the cells themselves (2), (3). The most common signaling molecules found in Gram-positive bacteria are unmodified and post-translationally modified oligopeptides (4), (5). These peptide pheromones control several different biological processes, such as natural genetic transformation, bacteriocin production, virulence, sporulation, and biofilm formation (1), (6), (7). Apparently, peptide pheromones are not produced by Gram-negative bacteria, which instead seem to rely on N-acyl derivatives of homoserine lactone for intercellular communication (8). Interestingly, very recently, a third type of signaling molecule, which appears to be present in both Gram-negative and Gram-positive bacteria, has been discovered. Structural studies on this molecule (AI-2) carried out in the *Vibrio harveyi* system suggest that it is a five carbon furanone that results from the spontaneous cyclization of 4,5-dihydroxy-2,3-pentanedione (9), (10). Taken together, studies on cell-cell communication indicate that most bacterial species are capable of secreting at least one type of signaling molecule (3). This fact suggests that the ability of bacteria to sense the presence of other bacteria has originated relatively early in their evolution, and that this property is very important for their adaptability and survival in nature.

3. REGULATION OF COMPETENCE DEVELOPMENT IN *S. PNEUMONIAE*

In *S. pneumoniae*, intercellular communication is used to regulate competence for natural genetic transformation (reviewed in 11) and production of bacteriocin-like peptides (12), (13). Natural competence, a physiological state in which bacteria are able to take up exogenous DNA, is found in both pathogenic and environmental bacteria, such as *S. pneumoniae*, *Neisseria gonorrhoeae*, *Helicobacter pylori*, *Bacillus subtilis*, and *Acinetobacter calcoaceticus* (14). Competent bacteria will recognize and bind naked double stranded DNA fragments present in their environment, and translocate these fragments in a single stranded form across the membrane into the cytoplasm (15). If a DNA fragment taken up by a competent cell recombines with the bacterial chromosome, it might permanently change the cell's genotype, hence the

name transformation. Recent studies that compare the rate of recombination in different species have revealed that exchange of DNA by homologous recombination (most probably via transformation) happens frequently in *S. pneumoniae*, and that the long-term evolution of the pneumococcal population seems to be dominated by recombination (16). In most naturally transformable bacteria, the competent state is not a constant property, but represents a transient physiological state (14).

In *S. pneumoniae*, a quorum-sensing mechanism consisting of an unmodified peptide pheromone, its secretion apparatus, and a two-component regulatory system (TCS), is used to regulate competence for natural genetic transformation (17), (18), (19), (20). However, competence development in streptococci also depends on other key factors, some of which are still poorly characterized. In the following sections, we describe in more detail how cell-cell signaling triggers a global change in gene expression in *S. pneumoniae* leading to transcription of the so-called late genes that encode proteins involved in binding, processing, uptake, and recombination of DNA.

3.1. Identification of the competence stimulating peptide (CSP)

In the middle of the nineteen sixties, it was discovered by Pakula and Walczak (21), and by Tomasz and Hotchkiss (22) that coordinated induction of competence takes place at a particular cell density in vigorously growing cultures of streptococci. It was also found that sterile filtered medium from a competent culture contained a proteinaceous compound termed competence factor (CF) that could substitute for cell density. When conditioned medium containing CF was added in small amounts to dilute cultures of streptococci, the competent state was immediately induced (21), (22), (23). This work probably represents the first example of a cell-density dependent process in bacteria controlled by a secreted signaling molecule.

Almost three decades later, the first competence genes, *comA* and *comB*, were identified. These genes encode a typical ABC exporter (ComA) and its accessory protein (ComB) (24), (18). Disruption of the *comA* gene by insertion-duplication mutagenesis made the mutant non-transformable, demonstrating that the ABC exporter is essential for competence development in *S. pneumoniae*. Interestingly, the mutant strain was no longer able to produce the competence factor, but would still respond to it if this activator substance was supplied from a wild type strain (24), (25). Judging from these observation it seemed likely that the ComAB transporter constituted the secretion apparatus of the competence factor. Members of the ABC transporter superfamily are easily identified by the presence of the Walker A and B motifs and other signature sequences located in the ATP-hydrolysing domain. These ubiquitous transporters translocate a wide range of substrates (*e.g.* inorganic ions, sugars, drugs, amino acids, peptides and proteins) across prokaryotic and eukaryotic membranes (26). Unfortunately, the nature of the substrate of an ABC-transporter often cannot be predicted from its

amino acid (aa) sequence alone. However, a few years after the identification of ComA, a new family of ABC-transporters was discovered and characterized (see below). Members of this family possess unique N-terminal proteolytic domains, and in every known case their substrates are peptides containing a double-glycine leader (27). By chance, it was discovered that ComA is a member of this new family, strongly indicating that its substrate, the competence factor, is a small peptide. On the basis of this information a successful protocol for the purification of CF was devised (17).

Aa sequencing of the purified signaling molecule showed that it is a cationic peptide consisting of 17 aa residues. Fortunately, it turned out that a synthetic version of this peptide, termed the competence stimulating peptide (CSP), has full biological activity (17). Synthetic CSP has made transformation of *S. pneumoniae* easy, effective, and reproducible, and is now used by most, if not all, researchers using pneumococcal competence for genetic studies.

3.2. Processing and secretion of CSP
3.2.1. A family of proteolytic ABC-transporters

Many secreted peptides and small proteins are synthesized with a double-glycine (GG) leader at their N-termini. Most of these proteins function as pheromones or substances with antimicrobial activity, the so-called bacteriocins (5), (11), (28), (29), (30). The peptide pheromones, which are used for intercellular communication, are so far found only among Gram positive bacteria, whereas bacteriocins with GG-leaders are common in Gram negative bacteria as well (31), (30). Double-glycine leaders were first discovered in precursors of ribosomally synthesized bacteriocins approximately ten years ago (32). Sequencing of regions flanking the genes encoding these bacteriocins revealed that they are often co-transcribed with, or located next to, genes encoding ABC-exporters and their accessory proteins (33), (34). It was therefore predicted that dedicated ABC-exporters constitute the secretion apparatus of bacteriocins with GG-leaders. This theory proved to be correct as mutations introduced in the structural genes of these ABC-exporters prevented secretion of the cognate bacteriocin (33), (34). Having identified the dedicated secretion apparatus, it still remained a mystery how the unique double-glycine leader was removed from the precursor during maturation of the bacteriocin. Aa sequence comparisons of different ABC-exporters of bacteriocins with GG-leaders revealed that all had related N-terminal domains of ~150 aa containing two conserved sequence motifs ($QX_4D/ECX_2AX_3MX_4Y/FGX_4I/L$ and $HY/FY/VVX_{10}I/LXDP$). The conservation of a cysteine in the first motif and a histidine in the second suggested that they could be active site residues in a cysteine protease specific for removal of GG-leaders. This was confirmed by demonstrating that a recombinant version of the 150 aa N-terminal domain was able to remove the GG-leader from a bacteriocin precursor *in vitro* (27). Presumably, all gene products containing GG-leaders are processed and secreted in the same way, *i.e.* by dedicated ABC-exporters containing N-terminal proteolytic domains (27), (30).

How did this unusual family of ABC exporters evolve? Interestingly, searches in sequence databases show that *Methanothermobacter thermautotrophicus*, which belongs to the Archaea, contains a homolog of the proteolytic domain. This homolog is about the same size as the proteolytic domain (~150 aa), and contains all the conserved active site residues. Based on this observation, it is tempting to speculate that the proteolytic domain of ABC exporters has originated by gene fusion between the transporter's structural gene and a gene encoding a cysteine protease similar to that found in the genome of *M. thermautotrophicus*.

3.2.2. The double-glycine leader

Based on the aa sequence of CSP, the gene (*comC*) encoding this peptide pheromone was identified (17). Sequencing of the *comC* gene revealed that CSP is synthesized as a precursor peptide, containing a double-glycine type leader at its N-terminal end. The ComC leader consists of 24 aa and contains the characteristic conserved glycine residues at positions -1 and -2 relative to the processing site. In addition, it has hydrophobic aa at positions -4, -7, -12, and -15, which is also typical for this kind of leader peptide (Figure 1) (17), (31). In general, double-glycine leaders are at least 15 aa long and usually contain the conserved aa motif described above. *In vitro* experiments with various synthetic versions of the 15 aa long GG-leader of lactococcin Gα, a bacteriocin produced by *Lactococcus lactis* LMG 2081, demonstrated that shortened versions of the leader were not processed by the proteolytic domain (unpublished results). This finding suggests that the 15 aa preceding the processing site are especially important for substrate binding and processing. Most likely the purpose of the double-glycine leader is to act as a handle which is grabbed by the proteolytic domain of the exporter. The leader is then cleaved off and the mature pheromone, or the bacteriocin, is translocated across the cytoplasmic membrane. The N-terminal domain of ComA contains the two conserved sequence motifs associated with proteolytic activity, and consequently belongs to the family of ABC-transporters described above. It is therefore highly likely that ComA removes the GG-leader from ComC concomitant with export.

3.3. The competence cascade
3.3.1. The ComCDE signal transduction pathway

Downstream of *comC*, genes encoding a histidine kinase (HK), ComD, and its cognate response regulator (RR), ComE, were identified (19). Together ComD and ComE constitute a typical TCS. Bacteria use TCSs to monitor external conditions, and to adjust their pattern of gene expression and physiology accordingly (35), (36), (37), (38), (39). A large variety of signals, including physico-chemical changes in the environment, are detected by membrane bound HKs, which activate their cognate cytoplasmic RR by transfer of a phosphoryl group. Most RRs function as transcriptional activators that stimulate expression of appropriate target genes in response to a signal (1), (2), (3), (4), (5). Against this background, it seemed plausible that the TCS downstream of *comC* constitute a signal transduction pathway regulating competence development in *S. pneumoniae*. Disruption of
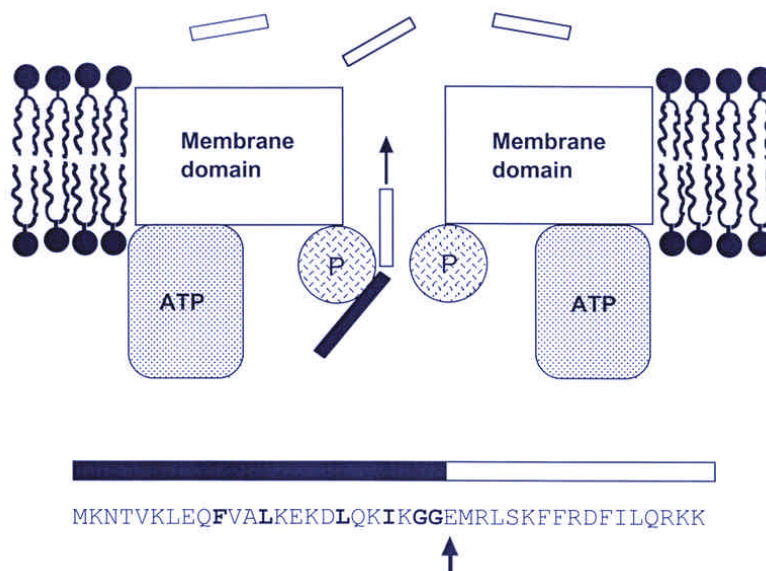
Figure 1. Model depicting processing and secretion of the competence stimulating peptide (CSP) by its dedicated ABC-exporter, ComA. The ABC–exporter operates as a dimer, and consists of three functional domains; an ATP-hydrolysing domain (ATP) providing energy for transport, a membrane domain that mediates translocation of the mature peptide across the membrane (open rectangle), and a proteolytic domain (P) that recognizes the double-glycine leader peptide (solid rectangle) and processes it as indicated by the arrow. Amino acid residues in the GG-leader of ComC that are conserved in most leaders of the GG-type are indicated in bold.

comE abolished both response to CSP and the spontaneous development of competence, demonstrating that ComE is indeed a key regulator of natural competence in *S. pneumoniae* (19).

3.3.1.1. ComD, the CSP receptor

Evidence that ComD is the CSP receptor was obtained by exploiting the strain-specificity of the CSPs produced by *Streptococcus gordonii* strains NCTC 7865 and Wicky (40). These two naturally competent streptococcal strains produce competence pheromones with different primary structures that are strain specific, *i.e.* the NCTC 7865 pheromone does not induce competence development in strain Wicky and *vice versa*. Furthermore, the *comD* genes of the two strains are highly homologous, but differ at their N-terminal ends. When introducing the *comD* allele of strain NCTC 7865 into the genome of strain Wicky, the resulting mutant responded to its own competence pheromone as well as to the CSP produced by NCTC 7865 (40). Thus, by tracing the CSP-specificity of the competence response in NCTC 7865 and Wicky to strain-specific alleles of *comD*, genetic evidence was obtained demonstrating that the HK ComD is the CSP receptor.

The ComD receptor belongs to the so-called orthodox kinases which are composed of a highly variable, membrane-spanning N-terminal sensor region and a more conserved, C-terminal kinase core domain that features the phosphoaccepting histidine as well as the various homology boxes H, N, D, F, and G. In a recent article by Grebe and Stock (41), a multisequence alignment of 348 kinase domains revealed that HKs can be divided into subgroups based on their degree of aa sequence identity. According to the criteria used, most of the HKs fell into one of eleven different subfamilies. Interestingly, ComD and most other peptide pheromone receptors fell into a subfamily designated HPK_{10}. Members of the HPK_{10} subfamily differ from most membrane-localized HKs in that they lack a D-box and contain only one asparagine in the N-box (41). In addition, they possess a characteristic sensor domain with 5-8 transmembrane segments. A comparison of highly homologous ComD receptors, which are specific for different but closely related pheromone types, show that they are most divergent in the first 60-100 N-terminal aa residues. This part of the ComD receptor, which contains two or three membrane-spanning segments, is likely to determine the specificity of the receptor-ligand interaction (40). The topology of the membrane domain of the ComD receptor has not been determined experimentally. However, computer programs, which have become quite reliable, predict that it has 6 or 7 membrane-spanning regions (42), (43). Presumably, these transmembrane segments and the loops connecting them will form a hydrophobic CSP-binding pocket at the outside of the cytoplasmic membrane. Alternatively, CSP could be inserted into the membrane in parallel to the transmembrane segments. Whatever the mode of receptor-ligand interaction, binding of CSP elicits a conformational change in ComD that results in dimerization, followed by autophosphorylation of active site histidines, in trans between the monomers (38), (39).

3.3.1.2. The ComE response regulator

As most RRs, ComE consists of an N-terminal regulatory domain and a C-terminal effector domain of

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comAB promoter        --GCAGTTGGG----12bp----TCATTTGGG--
comCDE promoter       --ACACTTTGG----12bp----ACAGTTGAG--
comX promoter         --GCAGTTTAG----12bp----ACAGAATGG--
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Figure 2. The DNA binding site of the response regulator ComE is an imperfect direct repeat motif found in the promoter regions of the *comAB* and *comCDE* promoters. *In vitro* electrophoretic mobility shift assays (EMSAs) have demonstrated that ComE binds to both of these motifs as a homodimer (44). Highly conserved nucleotide positions that probably are important for ComE binding are indicated in bold. A closely related sequence motif has been detected in the promoter regions of the *comX1* and *comX2* genes. EMSAs carried out with a dsDNA fragment corresponding to this motif have not been successful. Failure to demonstrate binding in this case could be due to substitutions of the two conserved thymines in the right repeat with two adenines (underlined positions), which presumably weakens considerably the interaction between ComE and the *comX* motif.

approximately the same size. In its phosphorylated state, ComD will act as a phosphodonor to ComE, which will catalyse phoshoryl transfer from the phosphorylated active site histidine in ComD to a conserved aspartate in its own regulatory domain (35), (36), (37), (38), (39). Most RRs function as transcriptional activators and have DNA-binding effector domains. This is also the case for ComE. Using electrophoretic mobility shift assays, it was demonstrated that ComE specifically binds to a target site consisting of two 9 bp imperfect direct repeats separated by a stretch of 12 nucleotides (Figure 2) (44). The spacing between corresponding bases in the first and second repeat is exactly two turns of the DNA helix. This suggests that ComE interacts with each 9 bp repeat and therefore binds the direct repeat motif as a dimer. It is not known if ComE can bind its target site in an unphosphorylated state (see 3.3.3), but phosphorylation of ComE will probably increase its efficiency as a transcription factor dramatically, and initiate transcription from promoters containing the direct repeat motif. Many RRs have a built-in autodephosphorylation mechanism that limits the lifetime of their activated state (39). Presumably, ComE also catalyses its own dephosphorylation (see 3.3.3), but it is not known whether this is a rapid or slow reaction.

3.3.1.3. An autocatalytic regulatory loop

The presence of a ComE-binding site in the promoter region of the *comCDE* operon readily accounts for the observed CSP induction of *comC*::*lacZ* transcriptional fusions in Rx strains (19) [For a comprehensive genealogy of *S. pneumoniae* strains, see 45]. Northern analysis confirmed that the *comCDE* genes are co-transcribed, and that transcription of this operon is strongly upregulated in response to synthetic CSP also in the R6 lineage (46), including the R6x strain, despite a previous negative report (20).

The presence of a ComE-binding site in the *comAB* promoter region (Figure 2) (44) is also fully consistent with CSP induction of a *comA*::*lacZ* transcriptional fusion (47). The observation that a

$comD^{T233I}$ mutation, which conferred constitutive competence, resulted in upregulation of *comAB* (48) provided independent evidence that *comAB* belongs to the ComE regulon. This conclusion was reinforced by transcriptome studies (49), (50). This regulatory loop generates an autoinduction mechanism that amplifies the response to CSP, resulting in higher rates of CSP synthesis and secretion, and an increased level of ComE-P inside the cells (11), (44). The biological significance of this autoinduction mechanism is not known, but it might serve to coordinate competence induction throughout the population.

Although the ComABCDE proteins together make up a quorum-sensing system that controls transcription of all competence (*com*) genes, transcription of the late *com* genes (see below) is not directly activated by ComE, but depends on ComX (see 3.3.2). Unexpectedly, ComX is encoded by two identical copies of the *comX* gene, designated *comX1* and *comX2* (47). A close examination of the promoter regions of the *comX* genes has revealed that they contain an imperfect direct repeat motif similar to the binding site of ComE (Figure 2), and for this reason expression of ComX1 and ComX2 is probably turned on by this RR. However, in the direct repeat motifs present in the *comX1* and *comX2* promoters, two conserved thymines are replaced by adenines in the right repeat sequence, suggesting that this potential ComE binding site will bind ComE-P with low affinity. This could be the reason why attempts to use electrophoretic mobility shift assays to demonstrate binding of ComE to this motif *in vitro*, have so far been unsuccessful (unpublished data). In comparison to a strong ComE-binding site, effective activation of transcription from a weaker binding site may require higher levels of ComE-P in the cell (see 3.3.3). Presumably, the autoinduction mechanism described above will increase ComE-P levels significantly, and it is possible that this abrupt increase in ComE-P concentration works as a switch to turn on ComX expression.

3.3.1.4. The *com* genes can be distributed into two classes, early and late

The first identified competence-regulated operon of *S. pneumoniae* was described as containing in addition to *recA*, which encodes a protein essential for homologous recombination, one (*i.e. cinA*) (51) or at least two (*i.e. cinA* and *dinF*) genes (52). A comparison of the kinetics of CSP-induced transcription of *comCDE* and *cinA-recA* revealed a strong induction of *comCDE*, only lasting for about 5 min (46). Then, while *comCDE* transcription was rapidly shutoff, induction of the *cinA-recA-dinF* operon was observed to occur with a ~5 min delay. Intriguingly, shutoff occurs while high concentrations of CSP are maintained in the culture medium. This pattern of transcription was confirmed by transcriptome studies using microarrays (49), (50) which showed that the whole competence regulon is made of two classes of *com* genes, early (*i.e. comCDE*-like) and late (*i.e. cinA-recA*-like) (Figure 3). The former class most likely relies on ComE~P for expression while the latter depends on the *comX*-encoded alternative sigma factor (see 3.3.2).
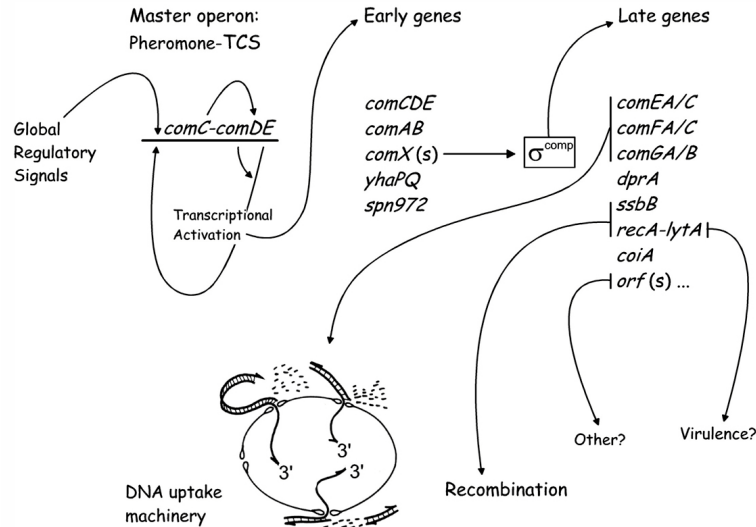
Figure 3. Pathway of competence regulation in *S. pneumoniae*. The pheromone precursor, ComC, is processed and exported by ComAB. The extracellular pheromone is detected by its specific receptor, the HK ComD, which, in turn, phosphorylates its cognate RR ComE. ComE~P drives expression of the early *com* genes, including *comX*. ComX (SigH) polymerase holoenzyme activates the transcription of late *com* genes. The latter encode the DNA uptake machinery (*i.e.* ComEFGs), recombination proteins (*e.g.* RecA), virulence proteins (*e.g.* LytA) and proteins of unknown functions. The DNA transport apparatus of *S. pneumoniae* (60) is likely to be very similar to that of *B. subtilis* (15). See text for explanations.

3.3.2. The ComX regulon

As mentioned above, transcription of the late *com* genes is not directly activated by ComE. No direct repeat motif similar to the ComE-binding site has been detected in the promoter regions of the late genes. Instead, these genes are expressed through a common regulatory element designated the combox or cinbox (53), (54), (55), (56), (57). The combox contains a conserved –10 sequence (TACGAATA) different from the Pribnow box, and therefore represents a typical binding site for an alternative sigma factor. As the binding site of this alternative sigma factor was identified before the protein itself, it was called ComX. Using a biochemical approach, Lee and Morrison (47) recently identified and characterized this sigma factor, and demonstrated that expression of the late *com* genes, but not the quorum sensing operons *comAB* and *comCDE*, were dependent on ComX (57).

Ongoing research in several laboratories aims at identifying all members of the ComX regulon, and elucidating their specific functions. Members of the ComX regulon can be distributed into four classes, two of which comprise genes dispensable for transformation (Figure 3). A major class of genes identified in several independent studies (49), (50), (53), (54), (55), (58), (59) encodes putative orthologues of the *B. subtilis* DNA uptake proteins. Recent data indicate that the proteins they encode carry out functions very similar to that in *B. subtilis* (60), *i.e.* binding of all types of double-stranded DNA present in the environment, processing and polarized transport of one strand into the cytoplasm (Figure 3; reviewed in 15). The second class of late *com* genes encode proteins, such as RecA and SsbB (Figure 3), involved in chromosomal integration of incoming homologous single strands by recombination. RecA plays a key role in this process as

recA knock-out mutants bind and transport, but do not incorporate DNA into the genome (52). Despite high basal level expression of *recA*, the observation of a ~20-fold reduction in transformants in a strain harboring a construct abolishing competence induction of *recA* highlighted the importance of the transient upregulation of *recA* (61). SsbB (CilA) (53), a candidate single-stranded DNA-binding protein, could facilitate the formation of a DNA-RecA filament, and thereby increase the efficiency of RecA-dependent homologous recombination. It was reported that a mutant with a defect in *ssbB* showed a 90% decrease in the rate of transformation (53). However, other observations suggest only a limited effect (Mathieu Bergé and J.P.C., unpublished data; Wei Wang and Donald A. Morrison, personal communication). The third class includes genes such as *lytA* (61), which has long been known to be dispensable for transformation (62). *lytA* encodes the major pneumococcal autolysin, which is potentially important for virulence (see 6.2). The last class comprises genes encoding proteins of unknown functions (Figure 3) (54), (58), (49), (50), (59).

3.3.3. The shutoff of competence

The transcriptional shutoff of the late *com* genes, as first observed for the *recA* operon (46) and confirmed by transcriptome analysis (49), (50), could be readily explained by targeted proteolysis of the competence transcription factor, similarly to the escape from competence in *B. subtilis*, as suggested by Tortosa and Dubnau (63). While transcriptome analysis indicated that *comX* mRNA rose and disappeared in a period of 15 minutes, and was maximal at 10 minutes (50), recent Western blotting showed that ComX existed transiently, with a maximum at ~15 minutes after addition of exogenous CSP, and totally disappeared ~8 minutes later

(Ping Luo and D.A.M., personal communication). These data would therefore be consistent with targeted proteolysis of ComX.

A similar hypothesis is not tenable for ComE which, unlike ComX, is stable for ~80 minutes following induction by CSP (44). The above mentioned *comCDE* transcriptional shutoff (2.3.1.4) and the transient failure of cells to respond to exogenous CSP ("blind-to-CSP period") for ~1.5 generation after spontaneous, as well as CSP-induced development of competence (64) remains poorly understood. It was first concluded that a late *com* gene product had a strong influence on the shutoff of ComE-dependent transcription because expression of a *comX::lacZ* transcriptional fusion was increased more than two-fold in a *comX* mutant and persisted during the blind-to-CSP period (47). Further results in the DNA microarray assay only partially confirmed this initial observation. Although in a *comX* mutant the CSP-induced expression of early *com* genes never returned to preinduction levels, unlike the situation in the wild type, expression continued but at only one-fifth maximal levels (50). It can be concluded from these results that CSP-induced expression of *comCDE*, *comAB* and *comX* loci is largely self-limiting. Self-limitation is responsible for ~80% of the shutoff, while completion of shutoff (*i.e.* elimination of ~20% residual expression) depends on *comX*. The finding that *rpoD* is induced 7-fold at competence (50) suggests a possible role of this induction in completion of the shutoff of the late *com* genes, as a significant increase in sigma A activity may contribute to ComX displacement. However, the significant (~80%) shutoff of early *com* genes occuring in a *comX* mutant cannot be explained simply on the basis of sigma factor displacement as previously suggested (47).

A proposal was made that ComE acts both as a transcription-stimulatory protein and as an inhibitor in different phosphorylation states (46). It was further suggested that the level of ComE within a cell determines whether it is sensitive to CSP, a high ComE concentration possibly preventing further induction (44). Alteration of competence shutoff in a ComE^{R120S} mutant (48) would be consistent with a direct involvement of ComE in transcription shutoff via dephosphorylation and/or multimerization. Interestingly, while the transcription kinetics of *comCDE*, *comAB* and *comX* were similar in wild-type cells, that of *comX* differed markedly from the other two in *comX* mutant cells (50). If the difference is significant, which is suggested by the reported reproducibility of expression patterns, this may indicate that different forms of ComE are involved in transcription of *comABCDE* and of *comX*. This would also strongly suggest that a ComX-dependent gene product somehow modifies the proportions of these different forms. A ComE-specific phosphatase, CEP, postulated by Alloing and coworkers (46) would be an obvious candidate for modifying the ComE/ComE~P ratio. However, none was evident among genes revealed through global genome analysis of the ComX regulon (49), (50), (59). Alternatively, the ComX-dependent gene product could be simply an enhancer of intrinsic, or ComD-stimulated, dephosphorylation of ComE. By analogy to the *B. subtilis*

Rap-Phr system of phosphatases regulated by extracellular peptides (4), this enhancer could be a peptide regulating phosphatase activity.

It has also been suggested that a repressor of *comCDE* is encoded by a *com* gene and binds an imperfect repeat (IR) that overlaps the transcription start of *comCDE* to shut down expression (44). However, upstream of *comAB* there is no IR ressembling that in *comCDE*, which makes it unlikely that the same repressor shuts down *comAB* expression, while *comAB* shutoff strictly parallels that of *comCDE* in both wild type and *comX* mutant cells (50).

A working model for shutoff combining several of these proposals and accounting for most of the above mentioned observations postulates that at low and intermediate ComE concentrations, a ComE-ComE~P complex allows transcription of *comABCDE*. At higher ComE concentrations, formation of a multimeric, fully phosphorylated form of ComE would occur, favoring *comX* transcription, while repressing *comABCDE* expression. This hypothesis takes into account the presence of imperfect ComE-binding sites upstream of *comX* (see 3.3.1.3). ComX-dependent synthesis of CEP would result in disappearance of ComE~P, thus preventing expression of all early *com* genes. At the same time, increased production of sigma A would favor displacement of ComX which, together with the instability of ComX (see above), would account for the shutoff of late *com* genes. High ComE concentration and the persistence of CEP would prevent further activation of ComE by activated ComD in wild-type cells, thus accounting for the blind-to-CSP period. Finally, the differential expression of *comX* observed by Peterson and coworkers (50) in *comX* mutant cells would be accounted for by the persistence of high ComE~P concentration, because CEP is not produced, while the decline in *comX* expression still observed in this context could be due to either intrinsic, or ComD-stimulated, dephosphorylation of ComE.

3.4. Control of basal level expression of *comAB* and *comCDE*

The early control of competence induction is not understood as well as later steps in the process. It was first proposed that CSP accumulates passively due to basal level expression of *comC*, possibly by transcriptional readthrough past the terminator of the tRNA^{Arg} encoded immediately upstream of *comCDE* (19), (64). An alternative model (65) involved regulated production of pheromone, which could be temporarily increased in response to changes in environmental conditions (e.g. modifications of nutritional or physical/chemical growth conditions).

With respect to the latter model, it has been known for a long time that aspects of metabolism or environment affect competence initiation. These include the requirement for $CaCl_2$, bovine serum albumine and high phosphate concentration, and the effect of the initial growth medium pH (low pH is inhibitory) or of replacement of the PO_4 supplement with maleate buffer (but not the substitution of maltose for glucose, as misquoted

previously) (reviewed in 11). Interestingly, a 10-fold reduction in the cell density at which competence first appeared was observed at an initial pH value of the growth medium of 8.25 compared to 6.79 (66).

Nevertheless, the exact role of these factors was not generally well defined. Hereafter, we review recent evidence suggesting that basal synthesis of *comCDE* and/or *comAB* is regulated, although the precise mechanism(s) of this regulation remain(s) to be established.

3.4.1. The oligopeptide permease

The observation that a strain carrying a duplication of the *comC* gene became competent at low pH provided the first indication that slight variations in *comCDE* expression can strongly affect competence development (19). Early spontaneous expression of *comCDE* was then observed in a mutant lacking all three oligopeptide-binding lipoproteins, *obl* (46). Oligopeptide uptake in *S. pneumoniae* involves a complex that includes a four protein ABC-permease, AmiCDEF, in addition to the three Obl (67). Analysis of the effect of varying inoculum size on the timing of competence development revealed that the *obl* mutant transformed at ~50-fold reduced cell density compared to wild type (46). A 30-fold reduction in cell density needed for competence development was also observed in a strain diploid for *comC*, suggesting that overexpression of *comCDE* could readily account for deregulation of competence in the *obl* mutant.

Although the oligopeptide permease is not required for responding to CSP (68), other observations suggested a possible role in modulating competence development (reviewed in 69). Lazazzera and Grossman (4) inferred from these observations that an additional peptide-signaling molecule could be imported to function intracellularly. However, an alternative model not invoking uptake of a signaling (*stricto sensus*) peptide was proposed (69). Peptide uptake, followed by peptidase digestion to release aa, and sensing aa pools through a global regulatory protein was suggested to provide information on nutrient availability (69). The oligopeptide permease could thus lead indirectly to modulation of the expression of several genes, including the *comCDE* operon.

3.4.2. *cup* mutants underline the importance of *comCDE* autoregulation

To better understand the early control of competence development, a genetic screen for mutants upregulating *comCDE*, or *cup* (for competence up) mutants, was set up by Martin and colleagues (48). Mutations were generated in a strain harboring a *comC::lacZ* transcriptional fusion by PCR mutagenesis of the *comCDE* region or by *in vitro mariner* transposon mutagenesis of the chromosome (see below). *cup* mutants were selected on the basis of increased beta-galactosidase activity on X-Gal-T⁻ plates, on which competence genes are normally not expressed.

Out of six independent *cup* mutations localized in the *comCDE* region, five resulted in single aa changes in the HK domain of ComD and one changed a residue

(R120S) located in the middle of the last alpha-helix at the C-terminal extremity of the receiver domain of the ComE RR (48). Martin and coworkers (48) identified a sixth mutation in *comD* (D299N) as responsible for the constitutive competence of a mutant strain, *trt*, isolated some thirty years ago (70). This mutational change was confirmed in a recent report (71). When assayed under conditions previously known to prevent competence of the wild type (*i.e.* acid growth conditions, or with maleate substituting phosphate, or in the presence of trypsin which digests circulating CSP), the *cup* mutants displayed various levels of transformation. The ComE^R120S turned out to affect viability in standard transformation medium, probably because high competence levels are incompatible with normal growth (48). As mentioned above (see 3.3.3), this mutant exhibited altered kinetics of competence shutoff. Altered shutoff is likely to affect cell viability because the global shift in protein synthesis accompanying competence induction (reviewed in 57) needs to be transient.

The dependence on circulating CSP for *comCDE* overexpression in the various *cup* mutants was evaluated by constructing *comA*⁻ derivatives of the mutants (48). Only the ComD^T233I protein resulted in similar transformability in the absence and presence of circulating CSP. Four other *comD* mutants, including *trt*, displayed 1000 to 10,000-fold reduced competence in the absence of circulating CSP. A divergent report on the lack of effect of a *comB*⁻ mutation transformation levels of the *trt* mutant (71) remains so far unexplained. In account of their results, Martin and coworkers (48) proposed that all ComD mutant proteins were able to self-activate in the absence of circulating CSP, but that the extent of this activation varied with the mutations. According to their hypothesis, ComD^T233I could be fully activated and mimic a phosphorylated HK in the absence of CSP, while the other ComD mutant proteins would be only partially activated and would require contact with CSP for full activation. A *comE* mutant (K38E) isolated by Echenique and colleagues (72) after nitrosoguanidine mutagenesis, using a screen for oxygen-independent transformability (see 3.4.5 regarding the effect of O₂), turned out to be CSP independent as well. The ComE^K38E protein was proposed to adopt an altered conformation, leading to permanent activation of the RR.

These results indicate that slight modifications in the interactions between CSP, ComD and ComE, can lead to dramatic changes both in the timing and in the conditions required for competence development. *comCDE* autoinduction could also play an important role in the early control of competence induction if CSP, once produced by a given cell, remains sequestered at the surface. This sequestration, which is seen in the R6 lineage (reviewed in 62), could lead to individual cell autoinduction, before the critical threshold for induction is attained in the culture medium. Release of CSP to the medium would then occur, when CSP-sequestering capacities become saturated. With respect to this hypothesis, it would be interesting to establish whether CSP release to the medium (typical for the Rx strain) or sequestration to the cell surface (as in the R6 lineage), is the rule for the species *S. pneumoniae*.

3.4.3. Limited CSP export capacity also controls *comCDE* autoinduction

Out of 52 independent *cup mariner* insertion mutants characterized by sequencing transposon-chromosome junctions, three were located immediately upstream of the *comAB* coding region (48), (73). Evidence was obtained that these insertions resulted in *comAB* overexpression (48). As they conferred a CUP phenotype, it was concluded that basal expression of *comAB* is limiting for CSP export capacity. Since limiting export impacts directly on the timing of competence development, Martin and coworkers (48) further inferred that the *comAB* operon constitutes a potential target for the regulation of competence. Thus, the fine-tuning of the early control of competence induction could be achieved by affecting the production and/or the stability not only of the CSP and of ComDE, but also of ComAB.

3.4.4. The CiaRH TCS and competence

The CiaRH TCS was first identified in a screen for beta-lactam resistance determinants in laboratory mutants (reviewed in 74). A *ciaH*^{T230P} change was found to lead to complete inhibition of competence (75). On the other hand, inactivation of *ciaR* or *ciaH* by plasmid insertion was reported to have no effect on competence (75). However, a careful analysis of *ciaR* mutants, isolated as spontaneous revertants of *ciaH*^{T230P} with a restored ability to transform, revealed that these mutants displayed competence under conditions inappropriate for the development of competence of the wild type (76). The isolation of four independent *mariner* insertions in *ciaR* in the above described screen for *cup* mutants provided strong independent evidence that CiaR affects the regulation of *comCDE* expression (48). A similar conclusion was attained by Echenique and coworkers (72) who screened for O$_2$-independent transformability (see 3.4.5 regarding the effect of O$_2$) following mutagenesis of the chromosome by plasmid integration, although astonishingly enough they obtained a unique *cup* mutant, the one harboring a plasmid in *ciaH*.

It is unknown whether CiaR affects *comCDE* expression directly or indirectly. Since CSP, in conjunction with ComAB and ComDE, exerts a positive feedback on *comCDE* expression (see above), CiaRH could affect the production and/or the stability of one of these elements or of some yet unidentified component of the feedback loop. If CiaR is directly involved in transcriptional regulation of elements of the competence regulatory cascade, its target could be *comCDE* or, as discussed above, *comAB*. In this connection, it was noticed that *comCDE* and *ciaRH* exhibit 83% identity over a 24 bp-long segment that includes the –10 promoter region of each operon, leading to the suggestion that this conserved sequence element may correspond to a CiaR-binding site (48).

It is also unknown which of CiaR or CiaR~P is involved in regulating *comCDE*. Martin and coworkers (48) pointed out that the CiaH^{T230P} change which prevents competence development most likely affects the phosphatase activity of CiaH, because an identical substitution (T253P) in the very same sequence context (SHELRTPL) in the *Escherichia coli* CpxA101 HK was demonstrated to alter the *in vitro* phosphatase activity of the HK toward its cognate RR, CpxR-P (77). This was interpreted as evidence that phosphorylated CiaR repressed competence (74). However, it is also possible that the CiaH^{T230P} context creates a special situation (*e.g.* cell wall alterations) that renders the mutant almost refractory to CSP. Consistent with the latter hypothesis was the observation that competence of the *ciaH*^{T230P} mutant was not (75), or only partially (74) restored by addition of CSP. Another puzzling observation is the finding that transcription of *ciaRH* increased 10-fold when K$_2$HPO$_4$ concentration in the transformation medium was raised from 1 to 50 mM, the transcription level in the latter case being similar to that in the CiaH^{T230P} mutant (76). If this is taken as an indication that the CiaRH system was activated, most likely through phosphorylation of CiaR, how could this be reconciled with the fact that 50 mM K$_2$HPO$_4$ corresponds precisely to the concentration required for development of competence, and therefore the concentration at which *comCDE* induction occurs? This might indicate that CiaR~P is not the active form of CiaR for repression of competence. The observation that inactivation of *ciaR*, but not of *ciaH*, resulted in *comCDE* overexpression (48) would be consistent with this hypothesis. It was concluded from these data that either the non-phosphorylated form of CiaR is active in repressing, directly or indirectly, *comCDE* or that, in the absence of CiaH, enough phosphorylation of CiaR occurs so as to lead to repression of *comCDE* (48). Clearly, further experiments are necessary to unravel the exact role and the mechanism of action of CiaRH in the regulation of competence.

Finally, as competence is dramatically reduced when cells enter stationary phase, it was suggested that CiaRH is activated during this period in wild-type cells (74). However, the observation that stationary phase repression of competence still occurs in strains harboring *ciaRH* knock-outs strongly suggests that other factor(s) are responsible for repression at this stage (Marc Prudhomme and J.P.C., unpublished data).

The signal(s) sensed through CiaH remain(s) unknown. However, two lines of evidence suggest that the CiaRH TCS plays a key role in cell wall polysaccharide biosynthesis (78). First, *ciaH* mutations allow cellular growth under conditions where cell wall biosynthesis is disturbed (*i.e.* during beta-lactam treatment) (74). Second, the presence of *uppS* among DNA fragments that specifically interact with CiaR suggests that the level of the *uppS*-encoded essential lipid carrier for peptidoglycan, teichoic acid and capsular polysaccharide precursors is under CiaR control (78). It is therefore possible that CiaRH integrates information pertaining to cell wall integrity and connects it to competence regulation. Independent evidence that alteration of peptidoglycan synthesis results in competence derepression is discussed below (see 3.4.6.4). Intriguingly, microarray analysis identified the *ciaH* gene as one of three spots still showing increased transcription 30 min after addition of CSP (49). Although the significance of this observation is unclear, it is possible that it is a reaction secondary to changes in the cell wall of

competent cells, which are known to be more prone to protoplast formation than uninduced cells (79).

3.4.5. Oxygen and competence

Echenique and coworkers (72) reported that O_2 limitation led to competence repression. They observed derepression of competence under microaerobiosis in the $comE^{K38E}$ mutant and in $ciaRH$ insertion mutants. They concluded from their results that the two TCSs, CiaRH and ComDE, mediate a response of *S. pneumoniae* to O_2, culminating in competence. Recently, they drew similar conclusions regarding the role of TCS02 (80), based on the finding that $rr02\text{-}hk02$ (called $micAB$) mutants transformed under microaerobiosis (81); see 3.4.6.1).

Martin and coworkers (48) pointed out that although *cup* mutations selected in T⁻ medium (see above) were also able to develop competence in T⁺ medium under microaerobic conditions, they should not be regarded as affecting O_2 regulation of $comCDE$ expression. They proposed to consider *cup* mutations, including those isolated by Echenique and coworkers (72) on the basis of "O_2-independent transformability", as affecting the control of basal level expression of $comCDE$ and resulting more generally in transformability under conditions suboptimal for competence development. Recent findings by Lacks and Greenberg (71) support this view. These authors reported that the lack of O_2 was not by itself sufficient to prevent competence development. Instead, they showed that the regime of microaerobiosis described by Echenique and coworkers resulted in severe reduction of pH because of the formation of large amounts of CO_2 (71). In agreement with previous reports on the effect of pH (22), (66), Lacks and Greenberg (71) demonstrated that the reduction of pH, and not the depletion of O_2, was responsible for the failure of competence to develop in wild-type strains under microareobic conditions. Further work would be required to establish whether competence of *S. pneumoniae* is really regulated by O_2.

3.4.6. Additional regulatory signals
3.4.6.1 TCS02

There are hints of influence from another TCS on competence development. TCS02 (80), also called 492hk/rr (82), $vicRK$ (83), or $micAB$ (81), is the *S. pneumoniae* orthologue of the $yycFG$-encoded essential TCS of *B. subtilis* (84). First, transformability in an $hk02$ ($vicK$) null mutant decreased by three-orders of magnitude (83). Second, Echenique and Trombe (81) reported that point mutations in $rr02\text{-}hk02$ affected competence regulation. They concluded from their data that alteration of the stability of the phosphorylated RR (detected in a $RR02^{D59A}$ mutant) or abolition of kinase activity (as observed in a $HK02^{L100R}$ mutant) allowed accumulation of $comCDE$ mRNA and competence development under microaerobiosis. However, intriguingly enough, Echenique and Trombe reported that insertional inactivation of $hk02$ conferred neither decreased transformability as observed by Wagner (83) nor the same competence phenotype as abolition of kinase activity in the $HK02^{L100R}$ mutant (81). Third, induction of $recA$ and of $ssbB$, two late *com* genes, was detected following induction of $rr02\text{-}hk02$ in a strain

harboring a copy of the $tcs02$ operon under the control of a fucose inducible promoter (Weonhye Bae and Martin Burnham, personal communication). The latter observations suggested that overexpression, but not depletion, of TCS02 could lead to competence induction. Further work may help reconcile these different results and clarify the role of TCS02 in the regulation of competence development.

Whatever the effect of TCS02 on competence induction, as in the case of CiaRH, it is not known whether TCS02 affects $comCDE$ expression directly or indirectly. Moreover, the signal(s) sensed through TCS02 remains unknown. Although Echenique and Trombe (81) inferred from their data that TCS02 was involved in competence repression under O_2 limitation, the conclusions of Lacks and Greenberg (71) regarding the microaerobic conditions used (see 3.4.5) apply here as well. Therefore it remains to be established whether TCS02 is in any way involved in O_2 sensing, or is more generally involved in integrating several cellular key parameters, such as DNA integrity and energy levels, as proposed by Wagner (83).

3.4.6.2. The ClpP stress response protein

Among the set of cup mutants (48), a mariner insertion was found to lie directly upstream of $clpP$ (85), a gene encoding the proteolytic subunit of the Clp ATP-dependent protease. This ubiquitous stress response protease plays an important role in regulation through proteolysis in both *E. coli* and *B. subtilis*. In the latter, ClpP is essential for expression of late *com* genes because in its absence the competence-specific transcription factor ComK is sequestered in an inactive form by the MecA/ClpC complex (86), (87). In contrast, *S. pneumoniae* ClpP acts negatively at the earliest stages of the competence regulatory pathway, possibly to prevent inappropriate expression of $comCDE$ (85).

3.4.6.3. Purine metabolism: purine pools as internal stress signals?

Several *cup* mutants were also found to carry insertions in three genes whose products likely function in purine metabolism. These are $purA$, $guaA$ and $guaB$, which encode putative adenylosuccinate synthase, GMP synthase and inosine-5'-monophosphate dehydrogenase, respectively (73). Interestingly, $guaA$ mutants were recently obtained in a screen for acid-resistance in *L. lactis* (88). Guanine nucleotide pools, notably (p)ppGpp were concluded to act as signals to determine the level of lactococcal stress response induction. We propose that, in *S. pneumoniae*, signals from purine metabolism are similarly used to adjust competence induction.

3.4.6.4. Membrane lipid composition and peptidoglycan synthesis

A major class of *cup* mutants is constituted by *mariner* insertions into the *cls* gene (48), (73). This gene potentially encodes the cardiolipin synthase which condensates two phosphatidylglycerol (PG) molecules to form cardiolipin (also called diPG) (reviewed in 89). Together with phosphatidylethanolamine, PG and cardiolipin are major phospholipids in bacterial

membranes. It is not known how the absence of cardiolipin results in competence induction. Two possibilities are envisioned. Alterations of membrane lipid composition could be directly sensed by a TCS. Alternatively, the modification of membrane lipid composition may affect DNA replication, which, in turn, would affect competence induction (see 3.5).

Another class of *cup* mutants corresponds to *mariner* insertions in the *pbp1b* gene (73). This dispensable gene encodes a high molecular weight penicillin-binding protein, with transpeptidase and glycosyltransferase domains (90), (91). Inactivation of *pbp1b* may affect peptidoglycan synthesis, which could be sensed through CiaRH (see 3.4.4).

3.5. Competence, a general stress response of *S. pneumoniae*?

The data reviewed above strongly suggest that information on nutrient availability (Obl and Ami; see 3.4.1), on cell wall (CiaRH and Pbp1b; see 3.4.4 and 3.4.6.4), on membrane lipid composition (Cls; see 3.4.6.4) and on stress conditions (ClpP and possibly purine metabolism; see 3.4.6.2 and 3.4.6.3) somehow affects competence development. These observations led to the proposal that competence is a general stress response of *S. pneumoniae* and that it may substitute for and play a role similar to the SOS response of *E. coli* (73). However the mechanism(s) and protein(s) connecting stress signals to *comABCDE* expression remain unknown. The CiaRH TCS is a likely candidate for sensing peptidoglycan alterations, as it has been suggested to play a key role in cell wall polysaccharide biosynthesis (78). Because of the similarities between CiaH and the CpxA HK of the CpxAR TCS of *E. coli* which is known to be involved in the control of envelope stress response (reviewed in 92), it has recently been speculated that CiaRH could be involved in sensing envelope stress signals rather than simply cell wall alterations (73).

Some effects could be indirect as in the case of the link between chromosome replication and competence induction postulated by Claverys and coworkers (65). These authors suggested that the co-localization of *comCDE* and *ori*, the putative origin of chromosome replication (93), offered an efficient means for adjusting the regulation of competence to replication as follows. In *E. coli*, it is well established that the frequency of initiation of chromosome replication at *ori* is dependent on growth rate and, therefore, on growth medium richness. Assuming this is also true for *S. pneumoniae*, any change in the frequency of initiation will impact directly on *comCDE* gene dosage, which, in turn, is likely to affect competence development, taking into account the previously mentioned effect of copy number on *comCDE* induction (see 3.4.1). This indirect mechanism could play an important role in connecting competence induction to growth rate and, consequently, to cell division.

A similar gene dosage effect could explain how inactivation of the cardiolipin synthase affects competence induction (see 3.4.6.4). As *E. coli* DnaA, the ubiquitous initiator of chromosome replication, interacts functionally with acid phospholipids such as cardiolipin (94), a modified cardiolipin content in *S. pneumoniae cls* mutants could impact directly on DnaA functioning. Any resulting alteration in the frequency of initiation at *ori* would, in turn, change *comCDE* gene dosage and *in fine* affect competence induction.

4. REGULATION OF COMPETENCE DEVELOPMENT IN OTHER STREPTOCOCCI

In recent years a number of *comCDE* alleles have been identified in different naturally competent streptococci from the mitis, anginosus, and mutans phylogenetic groups (95), (96), (97). The mitis group consists of the species *S. pneumoniae*, *S. mitis*, *S. oralis*, *S. infantis*, *S. crista*, *S, peroris*, *S. gordonii*, *S. sanguinis*, and *S. parasanguis*, whereas the three species *S. anginosus*, *S, constellatus*, and *S. intermedius* make up the anginosus group (98). Screening by PCR has demonstrated that the *comCDE* operon is present in isolates representing all species of the mitis and anginosus phylogenetic groups, except for the species *S. parasanguis* (unpublished data). This does not mean that all natural isolates of these species will transform well under laboratory conditions, which in fact they do not, but suggests that most of them are able to develop the competent state when subjected to the right growth conditions. In the mutans phylogenetic group, only the species *S. mutans* have so far been shown to be naturally competent and possess homologues of the ComCDE proteins (97). The highest diversity of *comC* alleles is found among strains belonging to the species *S. mitis*. More than 20 different *S. mitis* pheromones have been identified so far (unpublished data). In contrast, only two pheromone types (pherotypes), termed CSP-1 and CSP-2, predominate among pneumococci (96), (99), (100). The significance of the plethora of different CSPs produced by *S. mitis* strains is not understood. Presumably these strains grow together in complex communities, and together with other species make up biofilms in the nasal and oral cavities. *S. mitis* strains producing identical CSPs will sense each others presence if they grow close together in the biofilm, whereas strains producing dissimilar CSP will not. The apparent evolutionary drive towards increasing pheromone diversity within this species, suggests that it is advantageous for naturally competent streptococci to be able to discern between closely related and somewhat less related streptococci. The reason for this is not known.

Curiously, *comX* and the late genes seem to be more widespread in the genus *Streptococcus* than the *comCDE* operon. Orthologues of *comX* have been identified in *S. pyogenes* (101), and BLAST searches of other available streptococcal genome sequences indicate that they all contain the *comX* gene. The late genes and *comX* have even been detected in the genome of *Lactococcus lactis*. Are these competence genes non-functional relicts, or are bacteria such as *S. pyogenes* and *L. lactis* naturally competent after all? Perhaps competence in some streptococci traditionally considered to be non-competent has escaped detection because competence in these bacteria is turned on by some other mechanism than the ComCDE quorum sensing system.
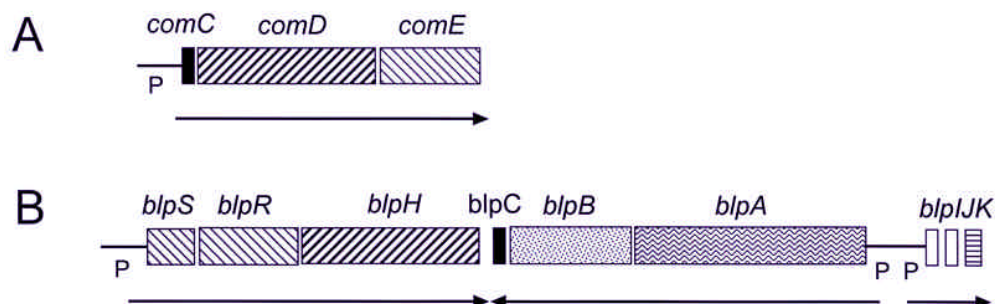
Figure 4. Organization of genes encoding two closely related quorum sensing systems regulating competence development (A), and bacteriocin production (B) in S. pneumoniae. The comCDE operon encodes the precursor (ComC) of the competence stimulating peptide (CSP), its histidine kinase receptor (ComD), and the cognate response regulator (ComE). The TCS BlpH and BlpR corresponds to ComD and ComE, respectively. The function of BlpS, which shows homology to the C-terminal half of BlpR, is unknown. The blpABC operon encodes the precursor (BlpC) of the bacteriocin inducing peptide (BIP), and its dedicated secretion apparatus consisting of the ABC-exporter BlpA and an accessory protein BlpB. The blpIJK genes encode two putative bacteriocin precursors (BlpIJ) and a possible immunity protein (BlpK). P indicates promoter regions and arrows represents mRNAs.

5. A CLOSELY RELATED GENE REGULATION SYSTEM IN *S. PNEUMONIAE*

A twin of the ComABCDE quorum-sensing system is present in *S. pneumoniae*. The two systems each consist of a peptide pheromone, encoded by *comC* and *blpC*, their dedicated secretion apparatuses ComAB and BlpAB, and the TCSs ComDE and BlpSRH (Figure 4). The more recently discovered system, BlpABCSRH, regulates production of several bacteriocin-like peptides of the class II type (Figure 4) (12), (13), (5). So far, it has not been demonstrated experimentally that any of these peptides possess antimicrobial activity. However, the striking similarity between the BlpABCSRH regulon and corresponding bacteriocin regulons thoroughly characterized in a number of lactic acid bacteria, strongly suggests that the function of the BlpABCSRH system is to regulate the production of several antimicrobial peptides in a cell-density dependent manner (28), (5). Some of the *blp* genes could encode the previously described pneumococcins and immunity systems (102).

The genes encoding the histidine kinase (*blpH*) and response regulator (*blpR*) are cotranscribed with a third gene of unknown function (*blpS*). This gene encodes a protein that shares significant sequence homology with the C-terminal DNA binding domain of BlpR, but lacks the N-terminal receiver domain (12). Thus, it is possible that BlpS and BlpR recognizes the same regulatory elements in the promoter regions of bacteriocin structural genes, and that BlpS therefore has a regulatory function. The peptide pheromones encoded by *comC* and *blpC* are both ribosomally synthesized as precursor peptides containing a double-glycine leader peptide at their N-terminal ends (see 3.2.2). Concomitant with export their double-glycine leaders are most likely removed by proteolytic domains in their respective ABC-exporters ComA and BplA. The mature pheromones, CSP and the bacteriocin inducing peptide (BIP), are both strain-specific. Allelic variation of the *blpC* gene, as in the case of *comC* (see 4), exists within the species *S. pneumoniae,* giving rise to at least four different BIPs (12), (13).

The two quorum-sensing systems appear to operate at different cell densities, at least in laboratory cultures of *S. pneumoniae*. The ComABCDE system autoinduces when the culture is in early logarithmic phase, whereas the BlpABCSRH system has been reported to autoinduce in late logarithmic phase (12). The two cell-cell signaling systems therefore seem to operate independently of each other, and cross-communication between them does probably not take place, neither at the pheromone nor at the HK level. BIP is completely unable to induce the competent state in *S. pneumoniae* strain Rx (unpublished data). This finding shows that BIP does not trigger autophosphorylation in ComD, and that BlpH cannot cross-phosphorylate ComE. Similarly, if CSP or ComD were able to cross-induce BlpH or BlpR, respectively, it would have been detected by de Saizieu and colleagues (12), when they used microarrays to identify the members of the regulon controlled by the BIP pheromone. The only possible connection between the two systems would be if export of BIP occurred via ComAB. As the transient increase in ComAB secretion capacity at competence (see 3.3.1.3) could contribute to the accumulation of higher levels of BIP in the growth medium, the critical threshold for induction of the BlpRH regulon could be attained earlier in competent than in noncompetent cultures.

6. CSP FOR COUNTING CELLS OR CELL-CELL SIGNALING?

6.1. Counting cells for genetic transfer or other purposes?
6.1.1. Counting donor cells for intraspecies exchanges

It was previously pointed out that regulation of competence for genetic transformation seems at first to be an unusual role for quorum sensing, as no product whose effectiveness for the producer cell would be compromised by dilution has been identified in *S. pneumoniae* (11). It was therefore proposed that the competence pheromone served to sense potential gene donors of the same species, based on the hypothesis that transformation evolved for the purpose of intraspecies gene transfer. Whatever the

mechanism of DNA release, accidental or competence-related (103), (104), the monitoring of cell density would ensure that competence develops when there is a high probability that DNA in the environment is from similar cells (4). Obviously, the existence of a competence-dependent, or even induced, mechanism of DNA release would improve the efficiency of intraspecies gene transfer. In this context, the existence of different pherotypes (see 4) raises an intriguing question. It was proposed that the different pherotypes have evolved to function as a sexual isolation mechanism (95), (63). As simultaneous colonization with more than one pneumococcal capsular type is not an uncommon phenomenon (reviewed in 105), nothing would prevent interpherotype exchange if DNA release is accidental. Therefore, the sexual isolation hypothesis is tenable only if DNA release is competence-dependent, or induced in a pherotype-specific manner. In addition, this release would have to occur with appropriate timing, as competence is only transient (see 3.3.3).

6.1.2. Counting recipient cells for horizontal transfer

As an alternative explanation, it was suggested that CSP served to sense potential recipients, rather than donor cells, to make sure that a large number of cells transform simultaneously (65). In this model, transformation would have evolved to permit the formation of new gene combinations by shuffling sequences through intra- as well as interspecies DNA transfer. As the likelihood for beneficial new combinations is low, high cell density would increase the probability for the occurrence of rare beneficial combinations (65).

6.1.3. Counting cells for competence-dependent virulence

The existence of connections between competence and virulence could also account for quorum sensing in competence development. The former possibility was first suggested by the identification of a fourth gene, *lytA*, in the previously characterized *cinA-recA-dinF* late *com* operon (61). This gene encodes the major autolytic amidase, which is known to be important for virulence (reviewed in 106). Additional support for the view that competence and virulence might be intimately connected came from the observation that *comDE* and *comGA-E* (but curiously not *comAB*) knock-outs were attenuated more than 8000- and 16000-fold, respectively, in a murine systemic infection model (59). In another study, a plasmid insertion *comD* mutant was found to be attenuated in bacteraemia and pneumonia models of infections, while a *comB* mutant was attenuated only in the bacteraemia model (107). Although the basis for virulence attenuation is unclear, several *com* genes, including *lytA*, could be important for virulence in murine models.

6.1.4. Counting cells for competence-dependent formation of biofilms

An alternative, non mutually exclusive hypothesis would be the existence of a link between competence of *S. pneumoniae* and biofilm formation, as suggested by the finding that at least *comD* is required for biofilm formation in *S. gordonii* (7). Thirty-year old reports describing increased agglutination of *S. pneumoniae*

competent cells (108, 109) suggest that competence may affect biofilm formation in *S. pneumoniae* as well.

6.2. Cell-cell signaling: the alarmone hypothesis

As discussed above, competence could confer increased genetic plasticity via DNA uptake and recombination (Figure 3) (65), contribute to virulence and possibly increase colonization ability, via the formation of biofilms. In every case, quorum sensing would ensure that low cell density does not compromise the process. An alternative possibility, in line with the hypothesis that competence constitutes a global stress reponse of *S. pneumoniae* (65), would be that the CSP is not only important for the purpose of monitoring cell density, but functions as an alarmone (48), *i.e.* an extracellular signal triggering an adaptive response to a changing environment.

7. PERSPECTIVES

The last decade has seen considerable progress in our understanding of the actors (ComABCDEX) and the mechanisms (including an autocatalytic regulatory loop) contributing to the synchronous development of competence in cultures of *S. pneumoniae*. The first decade of the new millenium will undoubtly see the unravelling of several of the open questions listed in this review, including the elucidation of the exact mechanisms for competence shutoff; a clear distinction between the *comAB* and *comCDE* operon as primary targets for the early control of competence development; the identification of protein(s) directly involved in this regulation; the elucidation of the role of orfs belonging to the competence regulon that encode proteins of unknown function (some of which might be involved in competence regulation); the establishment of whether there is some interplay between the two systems relying on extracellular peptide signaling (ComABCDE and BlpABCRH); a more precise description of the role of the various TCSs of *S. pneumoniae*, including CiaRH and TCS02, in the regulation of competence; and, finally, the identification of the various signals sensed through the oligopeptide permease and the TCSs. The latter information may help understand which forces contributed to drive the evolution of the extracellular peptide control of competence for genetic transformation in *S. pneumoniae* and other streptococci, as well as the significance of the plethora of competence pheromones existing among related streptococci.

8. ACKNOWLEDGEMENTS

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Send correspondence to: Dr Leiv Sigve Håvarstein, Department of Chemistry and Biotechnology, Biotechnology building, Agricultural University of Norway, P.O.Box 5040, N-1432 Ås, Norway, Tel: 47-64949464, Fax: 47-64947720, E-mail: E-mail: sigve.havarstein@ikb.nlh.no