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An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*

(ABC transporter/bacteriocin/peptide signal/*comC* gene)

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ABSTRACT **Competence for genetic transformation in *Streptococcus pneumoniae* has been known for three decades to arise in growing cultures at a critical cell density, in response to a secreted protease-sensitive signal. We show that strain CP1200 produces a 17-residue peptide that induces cells of the species to develop competence. The sequence of the peptide was found to be H-Glu-Met-Arg-Leu-Ser-Lys-Phe-Phe-Arg-Asp-Phe-Ile-Leu-Gln-Arg-Lys-Lys-OH. A synthetic peptide of the same sequence was shown to be biologically active in small quantities and to extend the range of conditions suitable for development of competence. Cognate codons in the pneumococcal chromosome indicate that the peptide is made ribosomally. As the gene encodes a prepeptide containing the Gly-Gly consensus processing site found in peptide bacteriocins, the peptide is likely to be exported by a specialized ATP-binding cassette transport protein as is characteristic of these bacteriocins. The hypothesis is presented that this transport protein is encoded by *comA*, previously shown to be required for elaboration of the pneumococcal competence activator.**

Bacteria respond to many changes in their environment by sensing specific small molecules. In cases in which the sensed compound is emitted by the cells themselves, a response can be attuned to the number of cells of the same species present in a locality. Such "quorum-sensing" mechanisms are known in a number of bacterial species. While traits known to be so regulated are quite diverse, including light emission (1, 2), virulence (3, 4), development (5), sporulation (6, 7), antibiotic production (8), plasmid-driven conjugation (9–12), and genetic transformation (13–15), one of the first shown to be mediated by release of a signal was competence for genetic transformation in *Streptococcus pneumoniae* (pneumococcus) (16).

Competence for genetic transformation in several bacterial species depends on achieving a specialized cellular state. As genetic transformation provides an efficient pathway for allele exchange, it is not surprising that it might occur in response to the presence of large numbers of cells of the same species, which are, *ipso facto*, potential gene donors. In pneumococcus, the competent state can be reached under relatively simple conditions, requiring neither stationary-phase cells (as for *Haemophilus*) nor late-logarithmic-phase cells (as for *Bacillus*), but simply exponentially growing cells at a critical, but rather low, cell density (16). The probable importance of this mechanism of genetic exchange is suggested by the evolution of new antibiotic-resistance genes by acquisition of gene segments from other oral streptococcal species (17).

Early studies of pneumococcal competence showed that its population dependence arises from the function of a protease-sensitive intercellular signal (16) and implicated as the key signal a small basic protein which could be extracted from cell walls of the strain R6 (18). A small protein was suggested by the observations that the signal sedimented slowly, if at all, but failed to pass through dialysis membranes and could be excluded from Sephadex G-25 gels. Since the description of the signal and of the outlines of the response of susceptible cells, most analysis of transformation in this species has been directed toward the DNA-processing pathway found in competent cells, without analyzing requirements for competence *per se*. Although subsequent research showed that protein synthesis was required for response to the signal (13) and that this response involves a temporary switch to the synthesis of a small set of new proteins (19, 20), the identity of the signaling molecule itself has remained unknown. We now describe the isolation and characterization of a peptide that is produced by pneumococcal cultures, induces competence development in dilute suspensions of growing cells, and is thus a key component in the coordination of competence within a culture.§

MATERIALS AND METHODS

Bacteriological Methods. Strains CP1200 [Nov^{S}ComA$^+$ (Nov, novobiocin), a transformable descendant of strain Rx], CP1415 (Nov^{S}ComA$^-$), and CP1500 (Nov^R) have been described (21, 22), as have the casein hydrolysate/yeast extract media used for growth and transformation assays (22, 23). Culture stocks for competence induction were prepared as exponentially growing cultures at about 3×10^7 cells per ml in growth medium supplemented with 0.1% yeast extract. Unless specified otherwise, competence was determined as Nov^R transformants obtained after exposure of cells to DNA in culture medium supplemented with 0.2% bovine serum albumin and 1 mM $CaCl_2$ (complete transformation medium, CTM) as described (21). The standard assay mixture (1–10 ml) contained ≈300,000 cells per ml, activator, and 1 μg of CP1500 donor DNA per ml. After incubation to allow development of competence and DNA uptake, samples were treated with DNase I at 10 μg/ml, incubated 90 min further for integration and expression of new alleles, and challenged with Nov (10 μg/ml) in agar plates as described (22).

Activator Purification. For large-scale preparations of activator, a 1.5-liter culture of strain CP1200 was grown at 37°C in CTM after addition of HCl to 5 mM. At an OD_{550} of 0.28, NaOH was added to a final concentration of 10 mM, and the cells were incubated for 40 min at 37°C for competence induction. After removal of cells from the competent culture by centrifugation in the cold and treatment of each liter of supernatant with 600 g of $(NH_4)_2SO_4$ for 18 hr at 4°C, the precipitate was collected by centrifugation at 7000 × *g* for 30 min and dissolved in 200 ml of buffer A (0.05 M sodium

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Abbreviations: CSP, competence-stimulating peptide; CTM, complete transformation medium; Nov, novobiocin.

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§The sequence reported here has been deposited in the GenBank database (accession no. U33315).

acetate, pH 5.2). Adsorption to 20 g of Amberlite XAD-16 (Supelco) was accomplished during 90 min at room temperature, and the beads were washed four times (30 min each) with 200 ml of 20% (vol/vol) ethanol in buffer A. Activator was eluted from the beads with 50 ml of 70% 2-propanol in 10 mM acetic acid, adjusted to pH 2 with HCl. After the volume was reduced to 20 ml *in vacuo*, 30 ml of buffer A containing 8 M urea was added, and the solution was applied to a cation-exchange column (Resource S; Pharmacia) equilibrated in buffer A containing 8 M urea. Bound material was eluted with a linear gradient of 0–1 M LiCl in buffer A containing 8 M urea. Active fractions were pooled and rechromatographed in the same way.

Active fractions were pooled, diluted 10-fold with 1% (wt/vol) orthophosphoric acid in water and applied to a C_2/C_{18} reverse-phase column (PepRPC 5/5; Pharmacia) equilibrated with the same solvent. Bound activator was eluted with a gradient of 0–100% 2-propanol in the same acid buffer. The sharp peak of active material was rechromatographed in the same way. The most active fractions from the reverse-phase column were diluted 10-fold in aqueous 0.1% trifluoroacetic acid, bound to the same column, and eluted with a 0–100% 2-propanol gradient in 0.1% trifluoroacetic acid. The two purest active fractions, as judged by SDS/PAGE (Pharmacia PhastSystem gel), were dried *in vacuo* and used for amino acid sequencing.

During purification, fractions were assayed for presence of activator in cultures of CP1200 as described above, with 250 μl of diluted cells in each assay mixture. After incubation with 1–5 μl of activator fraction (diluted when necessary to discern a peak of activity) and Nov^R DNA, 100 μl was spread on Nov selective plates.

Activator Structure and Synthesis. Edman degradation was done with an Applied Biosystems 477A automatic sequencer with an on-line 120A phenylthiohydantoin amino acid analyzer. Synthetic peptide was prepared by Research Genetics; the supplier reported that analysis by HPLC showed the product was 90% pure and that mass spectrographic analysis confirmed the predicted mass for the major component.

Activator Gene Identification. Degenerate *Eco*RI primers were designed from the amino acid sequences at the ends of the competence-stimulating peptide (CSP) and an expected Gly-Gly sequence adjacent to the initial residue (see *Discussion*): 5′-AATAGAATTCGGIGGIGA(A/G)ATG(A/C)GI(C/T)T-3′ and 5′-AATAGAATTCA(C/T)TT(C/T)TTIC(G/T)(C/T)-TGIA(A/G)IAT-3′. PCR was performed with these primers, CP1200 DNA, and Vent DNA polymerase as prescribed by the supplier (New England Biolabs). After 35 cycles with an annealing temperature of 38°C, products were analyzed by agarose gel electrophoresis. A fragment of the expected (80-bp) size was purified from the gel, cleaved with *Eco*RI, and ligated into plasmid pBluescript II SK(+) (Stratagene) by standard techniques. The DNA sequence between the primers was found to be: 5′-TGTCAAAATTCTTCCGTGATTTTAT-3′. A primer with this sequence (CF5) and a complementary primer (CF6) were synthesized.

CP1200 DNA (≈5 μg) was cleaved with *Rsa* I and ligated with *Hin*cII-digested and phosphorylated pBluescript II SK(+) (≈1 μg). To amplify flanking segments of the chromosome, a biotinylated polylinker primer (SK2, 5′-CCGCTCTAGAAC-TAGTGGATC-3′) was used with either CF5 or CF6 in PCRs with 0.3 μg of ligated DNA; after 35 cycles with an annealing temperature of 50°C, fragments of ≈700 bp (CF5/SK2) or ≈300 bp (CF6/SK2) were obtained. Both strands of each fragment were sequenced after purification from agarose gels using Dynabeads M-280 streptavidin as prescribed by the supplier (Dynal, Oslo); CF5, CF6, and SK2 were used as sequencing primers. Based on the sequences obtained, primers flanking the CSP gene were designed and used to amplify a fragment containing the CSP structural gene, which was then sequenced in the same way.

RESULTS

Purification of an Active Compound from Pneumococcal Cultures. The purification strategy relied on the principles described previously (18) but started with culture supernatants because strain CP1200 releases significant amounts of activator in soluble form (20, 24) and used chromatographic media and solvents appropriate for peptide isolation. Purification steps of ammonium sulfate precipitation, cation-exchange chromatography, and reverse-phase chromatography yielded about 2 μg of pure material of $M_r \approx 2000$, as judged from SDS/polyacrylamide gels (Fig. 1). In the final step, biological activity was found in three adjacent fractions; SDS/PAGE analysis of these and flanking fractions is shown in Fig. 1, illustrating the single-band purity achieved.

Structure of the Activator. About 2 μg of purified activator material was subjected to N-terminal analysis by Edman degradation; 17 clean cycles were observed, yielding the sequence of H-Glu-Met-Arg-Leu-Ser-Lys-Phe-Phe-Arg-Asp-Phe-Ile-Leu-Gln-Arg-Lys-Lys-OH. Since synthesis (see below) confirmed that this peptide stimulated competence in pneumococcus, we designate it CSP.

Identification of the CSP Gene and a Putative Prepeptide. Oligonucleotide primers designed on the basis of the peptide sequence were used for amplification, sequencing, and cloning of the corresponding part of the pneumococcal chromosome. In the relevant sequence (Fig. 2), codons corresponding to CSP comprised the second moiety of a larger gene, preceded by a ribosome binding site (AGGAG). Because it is the third gene identified in pneumococcal competence regulation, we named it *comC*. The 24 codons of the remainder of *comC* included 6 amino acid residues consistent with the peptide bacteriocin processing consensus, ending with Gly-Gly immediately preceding the processing site.

Induction of Competence and Complementation of a *comA* Mutation by Synthetic Peptide. The activity of synthetic CSP in eliciting competence for transformation was examined with two strains, CP1415, a *comA* mutant known to be deficient in activator production (21), and its transformation-proficient ancestor, CP1200. At a population level low enough to avoid spontaneous competence, both strains were induced to competence by the synthetic peptide. Peptide-induced competence reached a maximum by about 20 min at 37°C and then declined (Fig. 3). The *comA* mutant usually achieved somewhat (4-fold) higher competence than CP1200.

Stability of the Biological Activity of the Synthetic Peptide. Since earlier activator preparations purified from cell walls were labile (18), we explored the stability of the synthetic

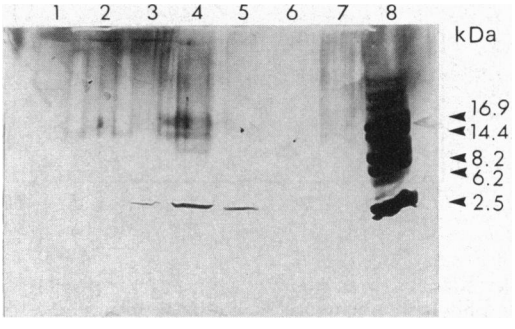

FIG. 1. Purification of active CSP from pneumococcal cultures. Fractions from the final column were analyzed by SDS/PAGE followed by silver staining. Only fractions 3, 4, and 5 exhibited competence-inducing activity, fraction 4 being the most active. Edman degradation of fractions 4 and 5 revealed identical sequences.

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AggagaTTTTATTATGAAAAACACAGTTAAATTGGAACAGTTTGTA 46
          M  K  N  T  V  K  L  E  Q  F  V
                                     F

GCTTTGAAGGAAAAAGACTTACAAAAGATTAAAGGTGGGGAGATGA 92
A  L  K  E  K  D  L  Q  K  I  K  G  G  E  M  R
   L  S        E  L        I     G  G              cons.

GGTTGTCAAAATTCTTCCGTGATTTTATTTTACAAAGAAAAAAGTAA 139
  L  S  K  F  F  R  D  F  I  L  Q  R  K  K  *
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FIG. 2. Sequence of the CSP gene, *comC*. Within the open reading frame encoding CSP, the initial codon of *comC* was assigned as the only ATG or TTG associated with a potential ribosome binding site (lowercase); residues of *comC* corresponding to CSP are underlined. The Gly-Gly processing-site consensus (cons.) for peptide bacteriocins according to Håvarstein *et al.* (25) is shown below the translation, to illustrate the matches at 6 of its 8 residues immediately upstream of CSP.

peptide by holding samples in aqueous solution at various temperatures before assaying residual inducing activity. In contrast to the half-life at 22°C of 1 hr reported previously (18), synthetic CSP was more stable, with half-lives of ≈8 min, 12 hr, and 9 days at 90°C, 37°C, and 4°C, respectively (data not shown).

Parameters Affecting Response to CSP. The conditions for competence induction by the synthetic peptide were explored. Only a few transformants were obtained by exposing cells to peptide at 0.5 ng/ml, whereas the highest yield, about 5% of cells transformed, was observed at doses of 30–1000 ng/ml (Fig. 4), and a monotonic dose–response characterized the intervening region. The sensitivity to CSP was thus not very different from the reported sensitivities to conjugation pheromone peptides and to homoserine lactones in photobacteria, with detectable reactions to levels near 10^{-10} M (1). Induction was optimal at pH 7.4–8.8, but the effectiveness of low levels of CSP declined below pH 7 (Fig. 4). The response to CSP at 100 ng/ml was independent of cell number over a wide range (Fig. 5), as would be expected if all cell-to-cell signaling components of the system had been obviated. Induction was observed with cells in all phases of growth except late stationary phase (Fig. 6). Although the choice of culture medium is often very important for achieving competence in pneumococcus, this seems not to be true for CSP induction of competence. So long as the pH was kept in the appropriate

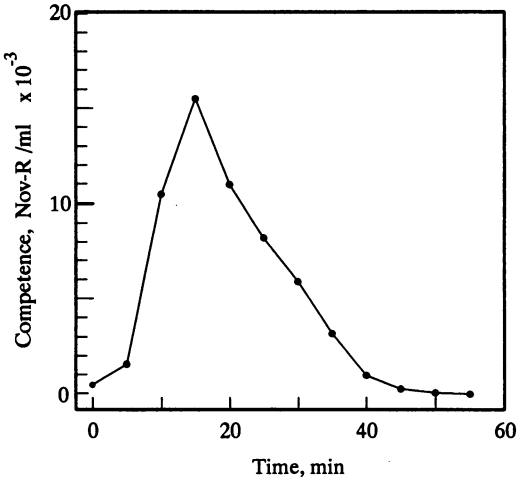

FIG. 3. Kinetics of induction of competence in pneumococcus by synthetic CSP. After addition of CSP (100 ng/ml) to CP1415 cells at 310,000 per ml in CTM, competence was assayed at the indicated times by exposure to DNA for 5 min and is expressed as the number of Nov^R transformants per ml. Data points are averages of duplicate samples. Cells were prepared by growth to an OD_{550} of 0.03 in medium supplemented with 0.1% fresh yeast extract, followed by 30-fold dilution into CTM for the assay reaction.

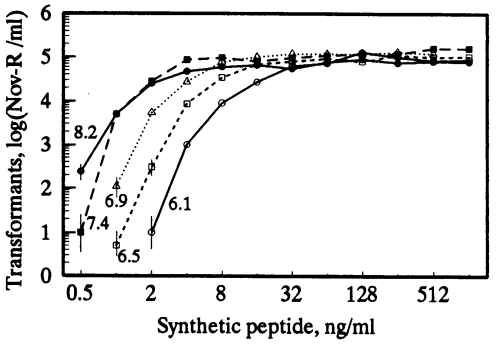

FIG. 4. Titration of competence-stimulating activity of synthetic CSP. Total Nov^R transformants were determined after a 150-min exposure of CP1415 to CSP and DNA in CTM adjusted to the indicated pH values. Total cells were 2.6×10^6 per ml.

range, CSP also induced high-level competence in the absence of the usual calcium and albumin supplements and in the three additional media we tested: Todd–Hewitt broth/yeast extract, brain–heart infusion, and Luria broth (data not shown).

DISCUSSION

Bacterial signaling by means of peptides is involved in a variety of processes. In *Myxococcus* fruiting-body development, the A signal is a mixture of peptides and certain amino acids (5). Peptide signals are believed to be involved in *Bacillus* sporulation (6, 7). Also in *Bacillus subtilis*, a 9- to 10-aa modified peptide and another uncharacterized peptide of similar size convey signals converging on the *srfA* operon required for induction of competence for genetic transformation (14, 15). Mating responses for plasmid conjugation in *Enterococcus faecalis* are mediated by peptide pheromones (9) which are 7–8 residues long. Oligopeptide permeases appear to be involved in the response to some of these peptide signals (26, 27), and there are indications that the *spo0K*-encoded oligopeptide permease may be important for action of one of the *B. subtilis* competence pheromones, while the *comP/comA* two-component response-regulator system carries information from the other (15).

The pneumococcal CSP described here is larger than the peptides mentioned above, and its target is unknown. However, in view of the peptide character of this signal, it is of interest that some mutations in oligopeptide permease substrate-binding proteins have been reported to alter the pattern

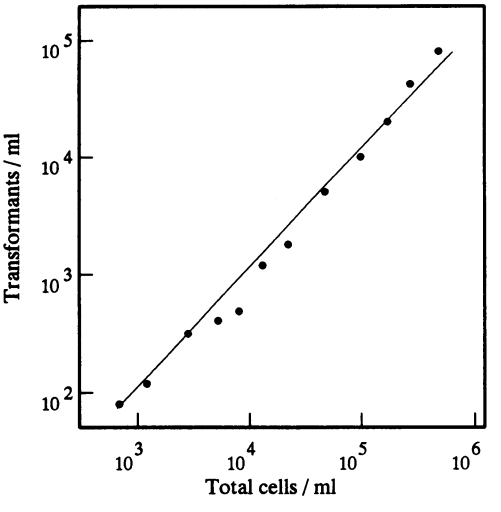

FIG. 5. Population independence of CSP response. Exponentially growing cells of strain CP1415 were exposed to CSP as in Fig. 4, after dilution to the indicated densities in CTM. Line indicates a slope of 1.0.

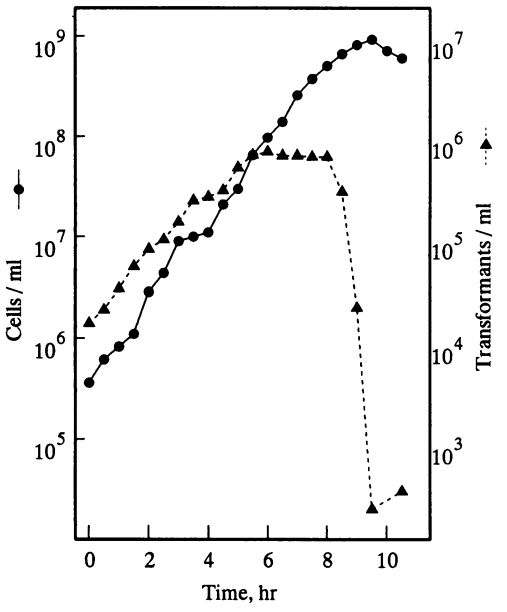

FIG. 6. Relation of CSP-induced competence to growth phase. CP1415 cells were diluted 1000-fold from a frozen stock culture (OD_{550} of 0.2) into CTM. Samples were taken at the indicated times during incubation at 37°C for determination of cell number (●) and of susceptibility to CSP (▲). Nov^R transformants were determined after incubation with CSP (100 ng/ml) and DNA (1 μg/ml) for 30 min, addition of DNase, and 90 min of expression.

of pneumococcal competence induction (28, 29). Pearce *et al.* (29) have postulated that these effects could reflect a direct interaction of an activator with the binding proteins. A critical role for these proteins in the CSP response seems unlikely, however, as CSP loss abolishes competence completely (21), whereas the reported phenotypes of severe permease mutations are more subtle, impairing transformation by only 50–90%. Also, CSP is rather large for an oligopeptide permease substrate (30), whereas AliAB and AmiA are known (28) to act in transport of small peptides (up to at least 7 residues). Finally, an essentially normal pattern of competence induction was observed (28) for triple mutants defective in all three oligopeptide-binding-protein homologues. In any case, the availability of synthetic CSP should now make the target more directly accessible.

The present results imply that the CSP primary translation product consists of 41 aa, of which the C-terminal 17 aa correspond to the secreted, biologically active CSP. Consequently, a 24-aa N-terminal leader peptide is removed from the primary product before, during, or after secretion of the CSP. The sequence of the leader peptide is not related to the N-terminal signal peptides that direct proteins across the cell membrane via the Sec pathway. However, it does clearly belong to a group of recently described leader peptides termed the double-glycine-type leader peptides (25). The following consensus sequence has been described for such leaders (numbered relative to the processing site): hydrophobic residue (−15), Leu (−12), Ser (−11), Glu (−8), Leu (−7), Ile (−4), Gly (−2), Gly (−1) (25). While hydrophobic residues almost without exception occupy the positions -4, -7, -12, and -15, only the Gly at -2 has been found to be universally conserved among these leaders. A Gly-Gly leader was first described for lactococcin A, a peptide bacteriocin from *Lactococcus lactis* (31). Peptide bacteriocins are ribosomally synthesized antimicrobial compounds with sizes between 2.3 and 9 kDa. The primary translation products of a number of additional peptide bacteriocins have now been described, most of them containing a Gly-Gly-type leader. CSP provides an example of a peptide with this type of leader whose biological function seems to be unrelated to that of bacteriocins, suggesting that such leaders might be more widespread than was previously thought.

It was shown by Hui and Morrison (32) that ComA, the protein required for competence induction in pneumococcus, is similar to several ABC transporters required for secretion of different bacterial proteins. It has therefore been suggested that ComA together with the ancillary protein ComB constitute an activator-specific export system (32, 33). Very recently, it was discovered that ABC transporters dedicated to the secretion of peptide bacteriocins with double-glycine-type leaders have conserved proteolytic domains at their N termini which remove the leader concomitant with export (34). The proteolytic domains of these transporters all share the two motifs Gln-Xaa_4-(Asp/Glu)-Cys-Xaa_2-Ala-Xaa_3-Met-Xaa_4-(Tyr/Phe)-Gly-Xaa_4-(Ile/Leu) and His-(Tyr/Phe)-(Tyr/Val)-Val-Xaa_{10}-(Ile/Leu)-Xaa-Asp-Pro. An N-terminal domain with this motif is found in ComA, strongly indicating that its substrate possesses a leader peptide of the Gly-Gly type. Together, previous results and the new data given here suggest (*i*) that CSP is the natural substrate of the ComA/ComB secretion apparatus and (*ii*) that the leader peptide of the CSP precursor is removed concomitant with export by the proteolytic domain of ComA.

It has long been known that pneumococci are capable of exchanging genetic information via extracellular DNA (35) and that the capacity for such exchange (competence) is evidenced in some cultures but not in others (36). While conditions for achieving competence in this species have never been clearly defined, the principal requirements have seemed to be (*a*) actively growing cells, (*b*) an absence of inhibitors, (*c*) a critical cell density (whose value varies considerably), and (*d*) calcium ions at about 1 mM. Although detailed comparison of the conditions required for spontaneous competence with those acceptable for CSP-induced competence are beyond the scope of this report, it is already evident that synthetic CSP elicits competence under an increased range of conditions and culture media. That conditions allowing this activation are quite broad, in contrast to the restrictive requirements for growth-induced competence, suggests that some of the requirements for the latter concern the spontaneous production and transmission of CSP, rather than the response to it.

Synthetic CSP may have significant practical applications. Although it is known that activator can be prepared and stored (18, 20, 24), most pneumococcal genetic studies have continued to rely on the "spontaneous" competence of a few rough laboratory strains. Indeed, competence is reported to be difficult to achieve in many primary clinical isolates, especially encapsulated strains (37, 38), although Yother *et al.* (24) showed that crude activator preparations could overcome the block to spontaneous competence in several encapsulated strains. It should be expected that readily available synthetic CSP may make competence more routinely accessible in many other pneumococcal lines, without the complications inherent in the use of a crude culture extract from a potential gene donor or the need to optimize conditions for signal elaboration. Finally, just as the structure of CSP should provide a handle for analysis of downstream response steps, so should it also provide tools to investigate the initiation of CSP synthesis and its relation to the timing of competence induction during the culture growth cycle.

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