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Competence for Genetic Transformation in Encapsulated Strains of *Streptococcus pneumoniae*: Two Allelic Variants of the Peptide Pheromone

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The nucleotide sequence of *comC*, the gene encoding the 17-residue competence-stimulating peptide (CSP) of *Streptococcus pneumoniae* (L. S. Håvarstein, G. Coomaraswamy, and D. A. Morrison, Proc. Natl. Acad. Sci. USA 92:11140–11144, 1995) was determined with 42 encapsulated strains of different serotypes. A new allele, *comC2*, was found in 13 strains, including the type 3 Avery strain, A66, while all others carried a gene (now termed *comC1*) identical to that originally described for strain Rx1. The predicted mature product of *comC2* is also a heptadecapeptide but differs from that of *comC1* at eight residues. Both CSP-1 and CSP-2 synthetic peptides were used to induce competence in the 42 strains; 48% of the strains became competent after the addition of the synthetic peptide, whereas none were transformable without the added peptides.

Genetic transformation not only is a valuable tool for molecular genetic analysis of *Streptococcus pneumoniae* (pneumococcus) but also appears to play a significant role in the evolution of this species, as in the assembly of mosaic antibiotic resistance genes containing blocks of information from other bacteria (6, 17) and in mediating a rapid mixing of alleles among natural populations (4). Yet, although transformation has long been described as a characteristic of the pneumococci, as a practical matter most studies involving this process have focused on a few laboratory strains, mainly descended from a single unencapsulated subclone (R36A) (2), for which optimal media and protocols were developed. Indeed, it has never been established just what proportion of pneumococcal isolates is transformable. This fraction has been difficult to assess, both because the pneumococcal capsule reduces or abolishes competence for genetic transformation (3, 5, 7, 14, 20, 25) and because the optimal conditions for competence vary between strains.

Competence for transformation in pneumococcus is not constitutive but is regulated by a quorum-sensing pheromone signal (23, 24). It was previously shown that culture supernatants from one strain, Rx1 (16), activate some other strains to competence and can induce competence in some encapsulated strains but not in others, including the type 3 strain A66 (25), even though an unencapsulated derivative of that strain is transformable (22). Following the recent identification of a small peptide from strain Rx1 with competence-stimulating activity (a competence-stimulating peptide [CSP]) (12), we reinvestigated the paradoxical behavior of A66, as a pure pheromone might be more effective than the crude culture supernatants used previously, but the results were still negative. We now trace the unresponsive character of strain A66 to the competence pheromone regulatory circuit itself and show that it reflects allelic variation of the pheromone gene in different isolates of this species.

Failure of A66 to respond to the CSP of Rx1. Strain Rx1 is descended from R36A, an unencapsulated transformable derivative of D39S, a type 2 clinical isolate that was employed in early studies on DNA biological activity (2, 20). In those studies, the donor DNA was prepared from the type 3 encapsulated strain A66. A66 was obtained from H. P. Bernheimer, and Rx1 was obtained from W. R. Guild (21). The responses to synthetic Rx1 pheromone peptide (TANA Labs, Houston, Tex.) for A66 and GP119, a type 3 encapsulated strain that we obtained by transformation of Rx1 with A66 DNA, were determined. Bacteria were grown in tryptic soy broth (TSB) (Difco), and 2 to 3 generations before stationary phase, cultures were frozen in 10% glycerol at −80°C. To carry out transformation, frozen cells were thawed and diluted 1:20 in competence medium (TSB [pH 8.0], 10% glycerol, 0.16% bovine serum albumin, 0.01% $CaCl_2$) containing the peptide pheromone (100 ng/ml) and transforming DNA (1,000 ng/ml) purified from strain DP1004 (*str-1*) (11). The transformation reaction was held 150 min at 37°C in a microtiter plate and then challenged with streptomycin in plates as described previously (19). The A66 strain was refractory to activation by synthetic CSP at levels that activated both Rx1 and the encapsulated strain GP119. Since the activation of GP119 indicated that the type 3 capsule of A66 is not responsible for the lack of response to CSP and since there is evidence for strain specificity of competence pheromones in *Streptococcus sanguis* (8–10), we investigated the possibility that a different allele of *comC*, the gene encoding the pneumococcal competence pheromone (12), was present in A66.

A66 carries a different *comC* allele. We used sequences near the gene in Rx1 (unpublished) to design primers for PCR amplification of a 230-bp product. With FI1 (5′AATGGTTT TTGTAAGTTAGC3′) and FI2 (5′ACAATAACCGTCCCAA

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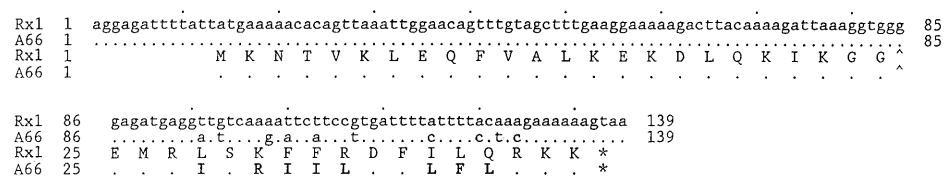

FIG. 1. Comparison of competence pheromone genes and their predicted translation products. Homologous chromosomal sequences from A66 and Rx1 are aligned. Above the top line of the sequences, periods show 10-base intervals. Within the sequences, identical residues are indicated by periods and termination triplets are indicated by stars. Amino acid residues 25 to 41 correspond to mature or synthetic pheromones. The Gly-Gly protease processing site (14) is marked by a caret.

ATC3′) (Cruschem, Glasgow, United Kingdom), 3 ng of A66 DNA template was amplified in a volume of 50 μl with 10 pmol of each primer for 30 cycles (93°C for 10 s, 72°C for 10 s, 52°C for 10 s) in 50 mM KCl–10 mM Tris-HCl [pH 8.0]–1.5 mM $MgCl_2$ with *Taq* DNA polymerase (Promega, Madison, Wis.). The PCR product was purified with the QIAquick PCR purification kit (Qiagen, Chatsworth, Calif.) and sequenced with [^{35}S]dATP and a modification of the Sequenase version 2.0 protocol (U.S. Biochemicals, Cleveland, Ohio). Denatured template (0.2 pmol) and primer FI1 or FI2 (10 pmol) were heated (94°C, 5 min), cooled, and chilled on ice; the labeling reaction nucleotides were reduced by half; and termination reactions were done for 3 min at 37°C. Products were resolved on 6% polyacrylamide gels which were dried and autoradiographed. The sequence, determined for both strands, revealed that A66 indeed carried a gene encoding a product similar to the CSP of Rx1 (Fig. 1). However, while the predicted gene product contained the same Gly-Gly-type leader peptide (12) as that in Rx1, processing at that protease site would produce a heptadecapeptide mature product differing from that in Rx1 at eight residues, including six conservative substitutions. The 10 base differences detected were all in codons for the mature peptide.

The A66 peptide induces competence in A66. As synthetic preparations of the Rx1 pheromone are biologically active, we tested the activity of a synthetic peptide of the sequence predicted for the mature A66 product (95% pure by high-pressure liquid chromatography analysis) (Neosystem, Strasbourg, France). A66, but not Rx1 or D39S, the encapsulated ancestor of Rx1, was activated to competence by the A66 peptide (Table 1). Although the new peptide tested had not been directly recovered from cultures of strain A66, we infer from these results that it does act as a pheromone in the regulation of competence in strain A66, that strains Rx1 and A66 encode different pheromones and correspondingly specific receptors, and that, therefore, they represent two distinct "pherotypes." We propose that the competence-stimulating peptides from strains Rx1 and A66 be named CSP-1 and CSP-2, respectively, and that the corresponding precursor genes be designated *comC1* and *comC2*.

Wide occurrence of Rx1 and A66 pherotypes. Noting that CSP-1 relaxes the environmental requirements for competence induction (12), that CSP-1 overcame the capsule barrier for GP119, and that CSP-2 did so for A66, we explored the range of CSP-induced transformability and of *comC* genes using a panel of 41 additional encapsulated strains collected during several decades, as listed in Table 1. Those strains marked with a G are 33 recent clinical isolates that were randomly chosen from an Italian collection (15). Others were the classic Avery strain D39S (the Rx1 parental strain [2]), SV1 (1), four American Type Culture Collection strains, and two strains from the collection of Glaxo Wellcome Verona (AII and 3496). The capsules were typed with the Pneumotest kit (Statens Seruminstitut, Copenhagen, Denmark), a system based on 12 pooled antisera. For DNA preparation, pneumococcal strains were grown to the end of log phase in tryptic soy broth, centrifuged in the cold, and lysed after resuspension in 0.15 M NaCl–15 mM EDTA–0.02% sodium dodecyl sulfate–0.01% deoxycholate–50 mg of proteinase K per ml. After 1 h at 56°C, DNA was extracted twice with chloroform, precipitated with

TABLE 1. Competence induction in encapsulated strains of *S. pneumoniae* by synthetic peptide pheromones

Strain	Capsule type or group	Allele	Competence induction[a] by:	
			CSP-1	CSP-2
D39S	2	*comC1*	+	−
G100	6	*comC1*	+	−
G405	6	*comC1*	+	−
G383	6	*comC1*	+	−
G388	9	*comC1*	+	−
G389	9	*comC1*	+	−
G374	17	*comC1*	+	−
G38	18	*comC1*	+	−
G46	19	*comC1*	+	−
G54	19	*comC1*	+	−
G99	19	*comC1*	+	−
G376	19	*comC1*	+	+
SV1	1	*comC1*	−	−
ATCC 6302	2	*comC1*	−	−
AII	2	*comC1*	−	−
G375	3	*comC1*	−	−
G396	3	*comC1*	−	−
G398	3	*comC1*	−	−
G399	3	*comC1*	−	−
ATCC 6303	3	*comC1*	−	−
ATCC 6305	5	*comC1*	−	−
G31	6	*comC1*	−	−
G394	6	*comC1*	−	−
G402	6	*comC1*	−	−
G393	9	*comC1*	−	−
G9	19	*comC1*	−	−
G40	19	*comC1*	−	−
G82	(29/34/35/42/47)[b]	*comC1*	−	−
G387	(24/31/40)[b]	*comC1*	−	−
A66	3	*comC2*	−	+
G48	3	*comC2*	−	+
3496	3	*comC2*	+	+
G386	6	*comC2*	−	+
G378	9	*comC2*	−	+
G86	(16/36/37)[b]	*comC2*	−	+
G5	(29/34/35/42/47)[b]	*comC2*	−	+
G385	(29/34/35/42/47)[b]	*comC2*	−	+
G60	3	*comC2*	−	−
ATCC 6307	7	*comC2*	−	−
G403	10	*comC2*	−	−
G363	10	*comC2*	−	−
G408	(24/31/40)[b]	*comC2*	−	−

[a] −, <1 transformant per 10^7 recipient cells; +, ≥1 transformant per 10^7 cells.
[b] Member of all capsule type groups.

TABLE 2. Competence induction in encapsulated strains of *S. pneumoniae* by two peptide pheromones

Allele	No. of strains induced to competence by:				Total no. of strains induced to competence
	CSP-1	CSP-2	CSP-1 or CSP-2	Neither peptide	
comC1	11	0	1	17	29
comC2	0	7	1	5	13
Total	11	7	2	22	42

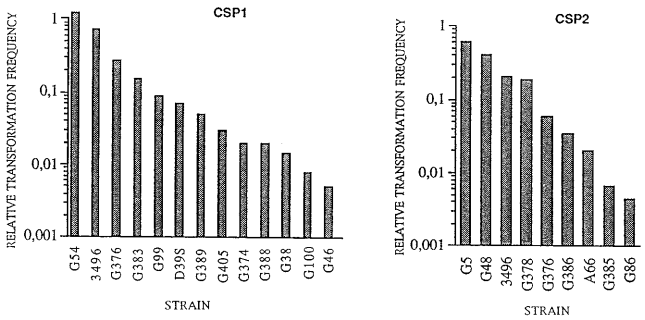

FIG. 2. Transformation frequencies of encapsulated strains of *S. pneumoniae* made competent with peptide pheromones. Transformation frequencies (expressed as percentages of transformed cells in a culture) were normalized to that of the control GP119 culture included in each set of cultures assayed. GP119 values varied from 0.06% to 0.65%. Results are shown for both CSP-1 and CSP-2.

ethanol, and dissolved in Tris-EDTA buffer. The nucleotide sequence of *comC* in each strain was determined for both strands as described above for A66, with the amplification products of two different PCRs used as templates. The sequencing gels were carefully analyzed for the presence of overlapping bands, which would indicate the presence of both alleles, but no such situation was ever detected in this study. The allele *comC2* was found in 12 additional strains, while *comC1* was present in the other 29 (Table 1). The nucleotide sequences of the two alleles were identical in all isolates tested, with the exception of nine *comC1* strains (G46, G99, G375, G376, G388, G389, G3903, G396, G399, and ATCC 6303) with a silent mutation (C95T) and three *comC2* strains (G48, G378, and G408) in which a G73T mutation caused the substitution of asparagine for lysine at position 20 in the leader peptide.

Induction of competence in encapsulated strains of *S. pneumoniae*. Both CSP-1 and CSP-2 synthetic peptides were used to induce competence in a total of 42 encapsulated strains (including A66) of *S. pneumoniae* (Table 1). Twenty out of 42 strains (48%) became competent after the addition of the competence peptides by the procedure described above (Table 2). None of the strains was competent in parallel cultures without added peptide. Two strains, G376 (*comC1*) and 3496 (*comC2*), responded to both CSP-1 and CSP-2, while each of the others responded only to the pheromone encoded by its own *comC* allele (Table 2). The level of induced transformation was up to 0.7% for a chromosomal point marker (Str-R) and, depending on the recipient strain, varied over a 200-fold range (Fig. 2). As we did not attempt to optimize conditions for each strain, this does not necessarily reflect genetic variation in the competence apparatus itself. Transformable strains belonged to a variety of serotypes, including 2, 3, 6, 9, 17, 18, and 19, with no obvious correlation between transformability and serotype. As we show here that encapsulated strains responded to CSP and were transformed under the culture conditions employed, these strains' lack of natural (not externally induced) competence must reflect a failure of CSP production; perhaps the point at which sensitivity to encapsulation is generally expressed is the initiation, by trace amounts of CSP, of the autocatalytic accumulation of activator (18).

Although genetic manipulation of encapsulated clinical isolates is essential for studying the molecular basis of pathogenicity and virulence of *S. pneumoniae*, encapsulated pneumococci were found difficult to transform, and the capsule has long been thought to reduce or block competence for genetic transformation. In this work, we show that (i) all pneumococci tested carried competence genes, (ii) there are two alleles encoding different competence pheromones in natural populations of pneumococci, (iii) the two pheromones are both active in inducing competence, and (iv) nearly half the encapsulated isolates of *S. pneumoniae* tested could be transformed with the synthetic pheromone peptides. By facilitating the genetic manipulation of encapsulated pneumococci, these results should open a new era in the study of *S. pneumoniae* as a pathogen.

Nucleotide sequence accession number. The nucleotide sequence reported here is assigned EMBL accession number X95385.

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