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Natural Competence in the Genus *Streptococcus*: Evidence that Streptococci Can Change Pherotype by Interspecies Recombinational Exchanges

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To map the incidence of natural competence in the genus *Streptococcus*, we used PCR to screen a number of streptococcal strains for the presence of the recently identified competence regulation operon, containing the *comC*, *-D*, and *-E* genes. This approach established that the operon is present in strains belonging to the *S. mitis* and *S. anginosus* groups, but it was not detected in the other strains examined. Competence is induced in *S. pneumoniae* and *S. gordonii* by strain-specific peptide pheromones, competence-stimulating peptides (CSPs). With its unique primary structure, each CSP represents a separate pheromone type (pherotype), which is recognized by the signalling domain of the downstream histidine kinase, ComD. Thus, all bacteria induced to competence by a particular CSP belong to the same pherotype. In this study, we identified a number of new pherotypes by sequencing the genes encoding the CSP and its receptor from different streptococcal species. We found that in several cases, these genes have a mosaic structure which must have arisen as the result of recombination between two distinct allelic variants. The observed mosaic blocks encompass the region encoding the CSP and the CSP-binding domain of the histidine kinase. Consequently, the recombination events have led to switches in pherotype for the strains involved. This suggests a novel mechanism for the adaptation of naturally competent streptococci to new environmental conditions.

Natural competence for genetic transformation is defined as the ability of a cell to take up free DNA from the surrounding medium. The DNA taken up can efficiently replace homologous regions of the recipient chromosome and thereby cause a permanent change in the cell's phenotype. This DNA processing pathway depends on the expression of a unique set of genes whose products are involved in binding, uptake, and integration of extracellular DNA. In naturally competent streptococci, the expression of these genes is not constitutive but is regulated by the gene products of the competence regulation operon. This operon consists of three genes encoding (i) the competence-stimulating peptide (CSP) precursor (ComC), (ii) a histidine kinase (ComD), and (iii) its cognate response regulator (ComE) (2, 6, 8, 19, 20). The CSP, which is secreted and processed by a secretion apparatus consisting of ComA and ComB (7, 9, 26), induces competence when its concentration in the medium reaches a critical level (17, 23, 24). At this concentration, the signal is perceived by the membrane-bound CSP receptor (ComD) (8), which probably activates ComE by transferring a phosphate group to the conserved receiver module of this response regulator (22). Presumably, the phosphorylated form of ComE acts at promoter sites for genes whose expression levels are upregulated during the development of competence.

So far, the amino acid sequences of four CSPs, two from *Streptococcus pneumoniae* (6, 20) and two from *S. gordonii* (8), have been published. In the study by Pozzi et al. (20), the sequence of the *comC* gene was determined for 42 strains of pneumococci. Two alleles (*comC1* and *comC2*), representing two pherotypes, were found. The gene products of these alleles (CSP-1 and CSP-2) turned out to be 53% identical at the amino acid sequence level. They were shown to be predominantly pherotype specific, but in two cases cross-induction was observed. The two CSPs (32% identity) from *S. gordonii* strains were also found to be pherotype specific. When possible cross-induction between two strains was tested, the number of transformants obtained was 5 orders of magnitude lower than that in the positive control. Nevertheless, this may have been above background, since no transformants were detected without CSP or with CSP-1 from *S. pneumoniae* (8). Together, these results show that CSP receptors are able to discriminate between closely related CSPs and that CSPs with 50% or less identity at the amino acid sequence level in general constitute different pherotypes.

In a study of phylogenetic relationships among 34 *Streptococcus* species, Kawamura et al. (11) divided the genus *Streptococcus* into the following six major clusters: the pyogenic group, the mutans group, the bovis group, the salivarius group, the mitis group, and the anginosus group (often called the *S. milleri* group). Most streptococcal species previously found to be naturally competent belong to the mitis group, but the phenomenon has also been observed in a few strains from the anginosus and mutans groups (1, 3, 5, 13–16, 18). In this communication, we present data which demonstrate that most or all strains belonging to the mitis and anginosus groups are naturally competent, whereas this property seems to be less common or even absent from the other groups. Furthermore, we report that *comCDE* operons sometimes contain a highly diverged sequence element in the region which determines the pherotype. These hypervariable segments must have arisen as the result of interspecies recombinational exchanges rather than by spontaneous mutation. Thus, naturally competent streptococci occasionally switch pherotypes when they take up and incorporate DNA from streptococci with a different pherotype.

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TABLE 1. Streptococcal strains screened for the presence of the *comCDE* operon

Phylogenetic group	Strain[a]	*comCDE* operon[b]
Mitis group	*S. pneumoniae* NCTC 7465^T	+
	S. mitis NCTC 12261^T	+
	S. mitis B5	+
	S. mitis B6	+
	S. mitis Hu8	+
	S. oralis NCTC 11427^T	+
	S. oralis ATCC 10557	+
	S. oralis DSM 20066	+
	S. gordonii NCTC 3165	+
	S. sanguis NCTC 7863^T	+
	S. crista NCTC 12479	+
Salivarius group	*S. salivarius* ATCC 13419	−
	S. salivarius NCTC 10235	−
	S. vestibularis NCTC 12166	−
Bovis group	*S. equinus* ATCC 9812^T	−
Mutans group	*S. mutans* NCTC 10449^T	−
	S. mutans NCTC 10832	−
	S. mutans NCTC 10919	−
	S. cricetus ATCC 19642^T	−
	S. rattus ATCC 19645^T	−
Pyogenic group	*S. dysgalactiae* NCDO 2023	−
	S. agalactiae NCTC 8181^T	−
	S. porcinus NCTC 10999	−
	S. canis DSM 20715	−
	S. iniae ATCC 29178^T	−
	S. zooepidemicus NCTC 4676	−
Anginosus group	*S. anginosus* NCTC 10713^T	+
	S. intermedius NCDO 2227^T	+
	S. constellatus NCTC 11325^T	+
	S. milleri NCTC 10708	+

[a] T, type strain.
[b] +, present; −, absent.

MATERIALS AND METHODS

Streptococcal strains and growth conditions. The designations, sources, and taxonomic statuses of the streptococcal strains used in this study are shown in Table 1. The taxonomic statuses of three clinical isolates, B5, B6, and Hu8, were determined by Statens Serum Institut (Copenhagen, Denmark). The strains were stored at −70°C and grown on 5% blood agar plates (BAP). The liquid medium used in transformation experiments was THB (Todd-Hewitt broth supplemented with 5% heat-inactivated horse serum). All incubations were done at 37°C in 5% CO_2.

Transformation assays. Competence was determined by obtaining streptomycin-resistant (Str^r) transformants after the exposure of cells to DNAs isolated from Str^r mutants (1,000 mg/liter) of the different strains included in this study. The following strains were tested for transformation: *S. anginosus* NCTC 10713, *S. constellatus* NCTC 11325, *S. intermedius* NCDO 2227, *S. milleri* NCTC 10708, and *S. gordonii* NCTC 3165. They were harvested from BAP and grown in THB for 18 h. Cultures were diluted 10-fold and grown for another 18 to 24 h until an optical density at 625 nm of about 0.8 was reached. At this stage, cultures were diluted 10-fold in THB, except for strains NCTC 10713 and NCTC 11325, which were diluted 100-fold to avoid endogenous competence induction. After incubation for 30 min, DNA from a Str^r strain (20 μg/ml) and synthetic CSP (200 ng/ml) were added. Depending on the growth rate of the strain tested, incubation was continued for 3 to 4 h before cultures were plated at appropriate dilutions on BAP with or without streptomycin (100 mg/liter). After incubation for 48 h, CFU were counted to determine the transformation frequency.

PCR sequencing, and analysis of sequence data. The preparation of genomic DNA, PCR, and subsequent sequencing of *comC* and *-D* genes were performed as previously described (8). The sequence alignment (see Fig. 1) and dot plots (see Fig. 2) were done with programs contained within the sequence analysis software package licensed from the Genetics Computer Group (University of Wisconsin, Madison). The Compare program, used to determine points of similarity between the two sequences compared in dot plots, was run with a window of 21 and a stringency of 17 bases.

Nucleotide sequence accession numbers. The nucleotide sequences reported here have been submitted to the EMBL database and given the following accession numbers: AJ000864 (*S. anginosus* NCTC 10713), AJ000865 (*S. mitis* B6), AJ000866 (*S. mitis* Hu8), AJ000867 (*S. constellatus* NCTC 11325), AJ000868 (*S. milleri* NCTC 10708), AJ000869 (*S. intermedius* NCDO 2227), AJ000870 (*S. gordonii* NCTC 3165), AJ000871 (*S. mitis* B5), AJ000872 (*S. sanguis* NCTC 7863), AJ000873 (*S. oralis* NCTC 11427), AJ000874 (*S. oralis* DSM 20066), AJ000875 (*S. mitis* NCTC 12261), and AJ000876 (*S. crista* NCTC 12479).

RESULTS AND DISCUSSION

Prevalence of the *comCDE* locus in major phylogenetic groups of the genus *Streptococcus*. It has previously been demonstrated that the *comCDE* operons of *S. pneumoniae* and *S. gordonii* encode components of a signalling pathway which regulates competence development in these bacteria (2, 6, 8, 19, 20). We decided to use PCR to look for this operon in other streptococcal species with the assumption that the presence of the *comCDE* genes could be used as a marker for natural competence. Unfortunately, comparisons of the available *comCDE* sequences revealed that they are quite divergent, making it difficult to construct PCR primers suitable for our purpose. We therefore chose to carry out PCR screening with primers complementary to the Arg- and Glu-tRNA genes previously shown to flank the *comCDE* operons in *S. pneumoniae* and *S. gordonii* strains (8, 19). By this approach, PCR fragments of the expected size (approximately 2.6 kb) were obtained for about half of the species tested. It should be kept in mind that detection of the *comCDE* operon depends on the flanking tRNA genes and that this operon escapes detection in any strain in which the competence regulation locus is organized differently. By sequencing about 350 bp from the 5′ end of each 2.6-kb fragment, the complete sequences of the *comC* genes of all positive strains and species were determined. For several of them (see below), the *comD* sequences were determined as well. The data in Table 1 show that in general, species belonging to the mitis and anginosus groups possess the *comCDE* operon, whereas it may be absent or less common in species belonging to the other major phylogenetic groups of the genus *Streptococcus*.

Natural transformation has long been described as a characteristic of species within the mitis group, such as *S. pneumoniae* (1), *S. gordonii*, and *S. sanguis* (5, 16). The high incidence of *comCDE*$^+$ strains in this group was therefore expected. The status of the anginosus group with regard to competence, however, has been more uncertain. Only a few reports have indicated that some strains assigned to the anginosus group are naturally competent (3, 13, 14). We were therefore surprised to discover that the *comCDE* operon is as prevalent among bacteria in this group as it is in the mitis group. In 1981, Perry and Kuramitsu (18) were able to demonstrate competence development in *S. mutans* HS6, GS5, and MT557. We examined three *S. mutans* strains (NCTC 10449, NCTC 10832, and NCTC 10919) and two other strains belonging to the mutans group for the presence of the competence regulation operon but found that they all were negative for *comCDE*. There could be several reasons as to why the results obtained by Perry and Kuramitsu differ from ours. (i) Only some species and/or strains within the mutans group are naturally competent, (ii) the development of competence in *S. mutans* strains is regulated by a different mechanism altogether, or (iii) the *comCDE* operon of *S. mutans* strains are not flanked by the tRNA genes described above. These matters are under investigation.

Diversity of pherotypes in the mitis and anginosus groups. Nine novel competence pheromones were discovered among

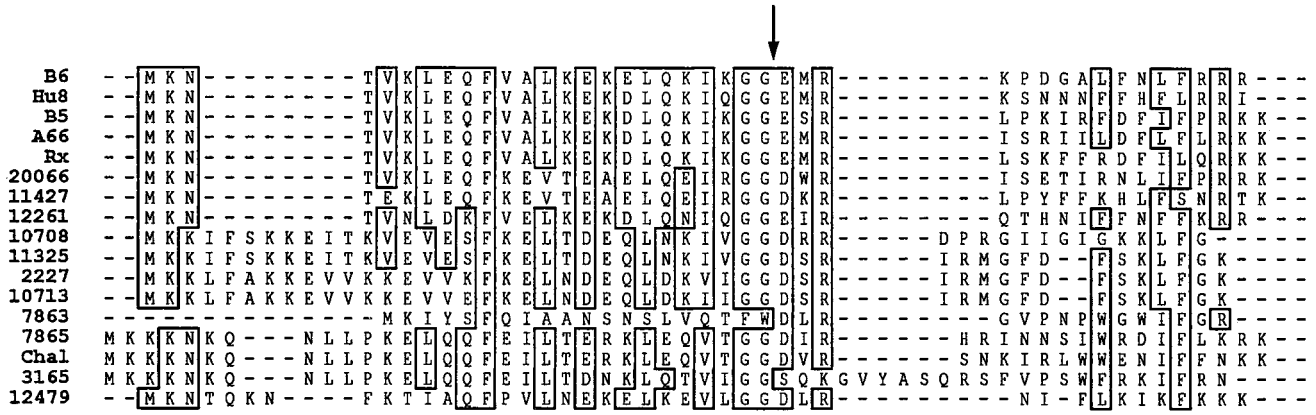

FIG. 1. Alignment of CSP precursors from different streptococcal strains. The N-terminal Gly-Gly leaders are cleaved from mature pheromones, as indicated by the vertical arrow. Amino acid residues that are identical or similar in at least 50% of the compared sequences are boxed. B6, *S. mitis* B6; Hu8; *S. mitis* Hu8; B5, *S. mitis* B5; A66, *S. pneumoniae* A66 (20); Rx, *S. pneumoniae* Rx (6); 20066, *S. mitis* DSM 20066; 11427, *S. oralis* NCTC 11427; 12261, *S. mitis* NCTC 12261; 10708, *S. milleri* NCTC 10708; 11325, *S. constellatus* NCTC 11325; 2227, *S. intermedius* NCDO 2227; 10713, *S. anginosus* NCTC 10713; 7863, *S. sanguis* NCTC 7863; 7865, *S. gordonii* NCTC 7865 (8); Chal, *S. gordonii* Challis (8); 3165, *S. gordonii* NCTC 3165; 12479, *S. crista* NCTC 12479.

the 11 strains examined from the mitis group, namely, the pheromones of *S. mitis* NCTC 12261, *S. mitis* B5, *S. mitis* B6, *S. mitis* Hu8, *S. oralis* NCTC 11427, *S. oralis* DSM 20066, *S. gordonii* NCTC 3165, *S. sanguis* NCTC 7863, and *S. crista* NCTC 12479 (Fig. 1). The pheromone of *S. pneumoniae* type strain NCTC 7465 turned out to be identical to the previously described pheromone from *S. pneumoniae* Rx (6, 20). Strain ATCC 10557, which used to be assigned to *S. sanguis*, has recently been reclassified to *S. oralis* (12). This strain was found to produce a competence pheromone unrelated to that of *S. sanguis* type strain NCTC 7863 but identical to the one produced by *S. oralis* type strain NCTC 11427. Thus, our data support the reclassification of strain ATCC 10557. Our results demonstrate that naturally competent species within the mitis group produce distinct CSPs, which in general have little homology to each other. Within this group, even strains assigned to the same species often make CSPs that have diverged to such an extent that they must be considered to constitute different pherotypes. In contrast, three species from the anginosus group, *S. anginosus* (NCTC 10713), *S. constellatus* (NCTC 11325), and *S. intermedius* (NCDO 2227), have identical CSPs, indicating that they are very closely related. Phylogenetic analyses based on 16S rRNA sequences and DNA-DNA hybridization experiments show that this is not the case; for instance, the phylogenetic distance between *S. anginosus* and *S. intermedius* is approximately the same as that between *S. pneumoniae* and *S. sanguis* (11, 25). Nevertheless, when DNA sequences corresponding to the region between the Arg-tRNA gene and *comE* (around 1,600 bp) were compared, we found that the sequences of *S. anginosus* and *S. intermedius* are 92% identical, whereas those from *S. pneumoniae* and *S. sanguis* have an identity of only 54%. This puzzling observation is best explained by assuming that the *comCDE* genes have been transferred horizontally between species in the anginosus group relatively recently. Frequent genetic exchanges among *S. anginosus*, *S. constellatus*, and *S. intermedius* could also explain why some workers have found that these strains are difficult to distinguish from each other by phenotypic tests (4, 25).

Properties of the CSPs. Figure 1 shows an alignment of CSP precursors, whose primary structures have been deduced from the nucleic acid sequences of their respective *comC* genes. The sequences of the CSP precursors of *S. pneumoniae* Rx and A66 and *S. gordonii* Challis and NCTC 7865 have been published previously (6, 8, 20). All of the precursors, except the one from *S. sanguis* NCTC 7863, contain leader peptides of the so-called double-glycine type. These leaders are removed concomitantly with export by dedicated ABC transporters containing N-terminal proteolytic domains (7). As their names indicate, the leaders are almost without exception cleaved behind two conserved glycine residues. Therefore, the amino acid sequences of mature secreted CSPs can easily be deduced when the sequences of their respective precursors are known. A few characteristics are shared by the CSPs aligned in Figure 1. They are all small (14- to 23-amino-acid) cationic peptides and in most cases contain a negatively charged N-terminal amino acid residue and a positively charged C-terminal end. The third amino acid residue from the N terminus is a highly conserved arginine, indicating that this positively charged residue is important for biological function. Based on these observations, we reasoned that the *S. sanguis* CSP precursor, which lacks a Gly-Gly leader, is cleaved between the amino acid residues at positions 20 and 21 (Fig. 1). A peptide (N-DLRGVPNPWGWIFGR-C) corresponding to the predicted CSP was synthesized and indeed had competence-inducing activity (results not shown), suggesting that this peptide is identical or at least very similar to the mature pheromone of *S. sanguis* NCTC 7863.

Sequencing data strongly indicate that members of the anginosus group are naturally competent. To confirm this, a synthetic version of the deduced CSP (N-DSRIRMGFDFSKLFGK-C) common for *S. anginosus* NCTC 10713, *S. constellatus* NCTC 11325, and *S. intermedius* NCDO 2227 was tested with each strain. As expected, the synthetic peptide (CSP/11325) induced competence in all three strains, but not in another member of the anginosus group used as a negative control (Table 2). This strain, *S. milleri* NCTC 10708, which has not yet been assigned to a particular species within the anginosus group, produces a different CSP. This peptide (N-DRRDPRGIIGIGKKLFG-C) induced competence in the *S. milleri* strain, but not in *S. anginosus* NCTC 10713, *S. constellatus* NCTC 11325, or *S. intermedius* NCDO 2227. Pherotype specificity was also demonstrated for the somewhat atypical CSP (N-SQKGVYASQRSFVPSWFRKIFRN-C) from *S. gordonii* NCTC 3165. This CSP, which lacks a negatively charged residue at the N terminus and has lysine instead of arginine at the conserved third position, was synthesized and tested with two streptococcal strains. It induced the competent state in *S. gordonii* NCTC

TABLE 2. Competence-inducing activities of different pheromones toward various strains

Strain	Frequency of transformants with indicated pheromone (%)					
	CSP/11325	CSP/10708	CSP/Challis	CSP/3165	CSP/7865	None
NCTC 11325	0.015	$<1 \times 10^{-4}$	$<1 \times 10^{-4}$		$<1 \times 10^{-4}$	$<1 \times 10^{-4}$
NCTC 10713	0.02	$<1 \times 10^{-5}$		$<1 \times 10^{-5}$	$<1 \times 10^{-5}$	$<1 \times 10^{-5}$
NCDO 2227	0.01	$<1 \times 10^{-5}$				$<1 \times 10^{-5}$
NCTC 10708	$<5 \times 10^{-5}$	0.08				$<5 \times 10^{-4}$
NCTC 3165			$<1 \times 10^{-5}$	0.01	$<1 \times 10^{-5}$	$<1 \times 10^{-5}$

3165 but had no effect on *S. anginosus* NCTC 10713. Furthermore, the two pheromones produced by *S. gordonii* Challis and NCTC 7865 were found to be inactive against *S. gordonii* NCTC 3165 (Table 2).

Pherotype switching in naturally competent streptococci. We have shown that a great diversity of pherotypes exists among naturally competent streptococci. Furthermore, all CSPs examined induced competence in the strains producing them as well as in strains producing identical pheromones. This pherotype specificity is of course due to the specific interaction between the CSP and its histidine kinase receptor (ComD). To compare *comC* and *comD* sequences from different pherotypes, we sequenced the regions between the Arg-tRNA and *comE* genes of the following strains: *S. milleri* NCTC 10708, *S. anginosus* NCTC 10713, *S. constellatus* NCTC 11325, *S. intermedius* NCDO 2227, *S. gordonii* NCTC 3165, and *S. mitis* B5, B6, and Hu8. Corresponding sequences have been published previously for *S. pneumoniae* Rx (19) and *S. gordonii* Challis and NCTC 7865 (8). Comparisons between *S. pneumoniae* Rx and *S. mitis* B5 and between *S. mitis* B6 and *S. mitis* Hu8 revealed that mismatches are more or less evenly distributed throughout the sequences, as would be expected if these closely related strains have evolved separate pherotypes by a gradual accumulation of random mutations (Fig. 2A and B). In contrast, the corresponding dot plots of *S. gordonii* NCTC 7865 versus *S. gordonii* Challis (Fig. 2C), *S. gordonii* NCTC 7865 versus *S. gordonii* NCTC 3165 (Fig. 2D), and *S. constellatus* NCTC 11325 versus *S. milleri* NCTC 10708 (Fig. 2E) revealed low-homology regions of 300 to 650 bp inserted between regions of high homology. The data in Fig. 3 show that in regions of low homology, there is 53 to 62% identity between the sequences compared, whereas in flanking regions the sequences are 92 to 100% identical. Considering these results, it is highly probable that the observed mosaic structures are due to interspecies recombinational exchanges, presumably by natural transformation. Interestingly, the inserted sequences encompass the region of the *comCDE* operon which determines the pherotype. In all three cases, the upstream crossover sites are located within the *comC* gene, close to the beginning of the coding region of the mature CSP. The location of the downstream crossover site varies, but in each case it is within the coding region of the membrane domain of the receptor (Fig. 3). Thus, the mosaic block observed in each case includes regions encoding the CSP and the part of ComD postulated to be involved in CSP binding. Taken together, our results demonstrate that the following two distinct pathways may give rise to new pherotype variants in streptococcal species: (i) accumulation of point mutations and (ii) horizontal gene transfer.

Virulence in *Staphylococcus aureus* is regulated by a signal transduction pathway similar to the ComCDE system. The components of that pathway are encoded by the *agr* locus and consist of a cell-density-dependent peptide pheromone and a two-component regulatory system. In a recent study by Ji et al. (10), three groups, corresponding to pherotypes, were identified among the strains of *Staphylococcus aureus* examined. Reminiscent of what we have described above, blocks of sequence divergence in the region of the *agr* locus responsible for the pherotype (i.e., ligand-receptor interactions) were observed. To explain how ligand and receptor could have diverged in concert, they suggested the involvement of some sort

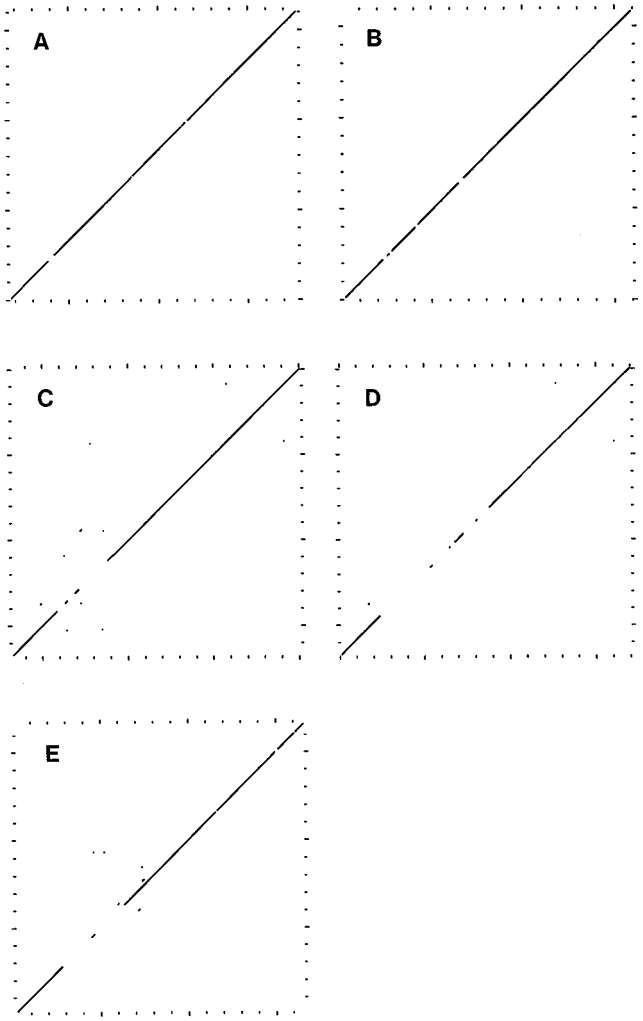

FIG. 2. Comparisons of nucleic acid sequences encompassing the promoter region and the *comC* and *comD* genes (approximately 1,600 bp) from different streptococcal strains. The comparisons were visualized graphically as dot plots. Each tick on an axis represents 100 bases. The following pairs of sequences were compared: *S. pneumoniae* Rx and *S. mitis* B5 (A), *S. mitis* B6 and *S. mitis* Hu8 (B), *S. gordonii* NCTC 7865 and *S. gordonii* Challis (C), *S. gordonii* NCTC 7865 and *S. gordonii* NCTC 3165 (D), and *S. constellatus* NCTC 11325 and *S. milleri* NCTC 10708 (E).

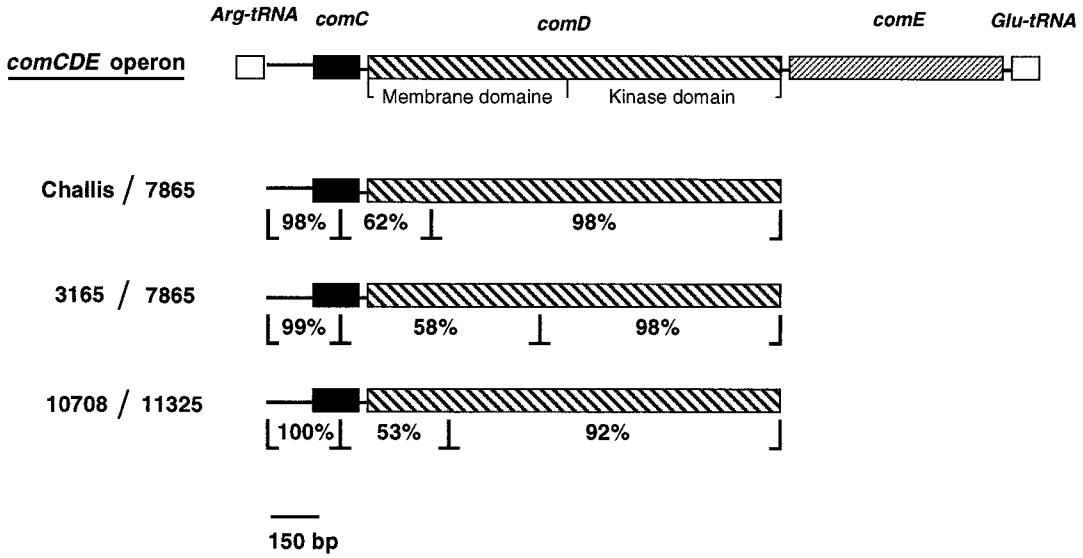

FIG. 3. Mosaic structures of *comC* and *comD* genes revealed by comparing the regions between the Arg-tRNA and *comE* genes of several related streptococcal strains. The nucleic acid sequences (approximately 1,600 bp each) were compared in pairs as indicated. Challis/7865, *S. gordonii* Challis against *S. gordonii* NCTC 7865; 3165/7865, *S. gordonii* NCTC 3165 against *S. gordonii* NCTC 7865; 10708/11325, *S. milleri* NCTC 10708 against *S. constellatus* NCTC 11325. Regions of high and low homology are indicated by brackets, and the percent identity is shown for each region. The low-homology regions are believed to have been introduced by recombination.

of cassette-switching mechanism or hypervariability-generating system. We propose that coevolution of pheromone and receptor in the *agr* system occurs by the same evolutionary mechanism as that demonstrated for the *comCDE* system, namely, interspecies recombinational exchanges. Since *Staphylococcus aureus* has been reported to be naturally competent (21), it is even possible that such gene exchanges take place by natural genetic transformation.

What then is the biological significance of pherotypes? Too little is known about the biology of natural competence and the circumstances which lead to gene exchanges between streptococci in their natural habitats to answer this question. Nevertheless, we now realize that pherotypes divide competent streptococci into bacterial populations and that communication can take place only within, not between, populations. Our results also show that it is possible for streptococci to overcome this restriction by undergoing gene replacements that lead to a change in the pherotype. From the high frequency of mosaic structures found so far, pherotype switching seems to occur relatively often among naturally competent streptococci, suggesting that such changes are selected under certain growth conditions. In summary, pherotype switching may be a biologically important mechanism which contributes to interspecies genetic exchange by making it possible for different species to be induced to competence by the same CSP.

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