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Genetic Diversity of Competence Gene Loci in Clinical Genotypes of *Streptococcus mutans*

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The frequencies of 21 competence genes were analyzed in 94 genotypes of *Streptococcus mutans*. These include those of a main regulatory system (*comCDE*), structural, and other regulatory orthologues identified in the genome of strain UA159. PCR and Southern blot analysis revealed that all genes are widespread within the species.

Streptococcus mutans are the major pathogens of dental caries, a biofilm-dependent infectious disease. These organisms are able to prevail in the complex microbial community of the oral biofilm in the presence of sucrose, under extremely low pHs responsible for tooth demineralization, and can physiologically adapt to the stressful conditions to which the cariogenic biofilm is exposed. Several of these processes involve two-component signal transduction systems (TCS). The most-studied TCS of *S. mutans* is the quorum-sensing system *comCDE* that regulates genetic competence (12) and also has been shown to play a role in biofilm formation and acid tolerance (10–12). Other regulatory (*comX1*, *mecA*, *ciaH*/*R*, and *LuxS*) genes appear to be involved in competence via a complex net of signals that may regulate structural genes involved in the early and late events of competence, including DNA binding, transport, processing, and recombination (16). These genes also may play a role in biofilm growth and structure (14, 20, 23, 27). The genetic diversity of the components of competence is unknown. In contrast to *Streptococcus pneumoniae* and many commensal streptococcal species of the oral cavity, e.g., *Streptococcus gordonii*, the frequency of *S. mutans* transformation is low, and the majority of isolates appear not to be transformable in vitro (17, 25). In the present study, we characterized the genetic organization of 11 chromosomal loci of regulatory and structural genes with known or putative roles in competence in a collection of *S. mutans* genotypes isolated from children during the initial phase of colonization.

A total of 94 *S. mutans* genotypes were analyzed. Fifty genotypes were isolated from 14 6- to 24-month-old children, 2 of whom presented with initial caries lesions (8). The other 44 genotypes were isolated from 35 12- to 30-month-old children in a separate study of the same population (13). Carious lesions were detected in 15 of these children. The genotypic identities were determined by arbitrarily primed PCR as described in previous studies (8, 13). Strains were grown from frozen stocks in Todd-Hewitt or brain heart infusion broth at 37°C in an atmosphere of 10% CO_2 and 90% O_2.

Genomic DNAs were purified from 1.5 ml of culture by using a MasterPure DNA purification kit (Epicenter Technologies, Madison, Wis.) as recommended by the manufacturer. A total of 21 genes from 11 loci within the genome of *S. mutans* strain UA159 (http://www.genome.ou.edu) were analyzed by PCR (Table 1). The gene organization within the UA159 chromosome and the position of the respective primer sets used for screening are shown in Fig. 1. Table 2 shows sequences of primers specific for each locus. PCRs were performed in volumes of 50 μl (200 μM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 0.3 μM concentrations of each upper and lower primer, and 1.25 U of *Taq* DNA polymerase [Invitrogen]). The thermal conditions varied slightly for each locus analyzed and included 35 cycles of denaturing at 95°C for 45s, annealing from 50 to 52°C for 1 min (Table 2), and extension at 72°C for 2 min. The genomic DNA of strain UA159 was used as a positive control in all PCR baths. *Streptococcus sobrinus* strain 15JP1 was used as a negative control. PCR products were resolved (8 V/cm in Tris-borate-EDTA) in 0.8% agarose gels and stained with ethidium bromide. To confirm gene absence in the PCR-negative genotypes, Southern blot assays were performed using the amplicons obtained from the control UA159 as probes. Restriction maps are shown in Fig. 1. After digestion of 3 μg of genomic DNA at the appropriated conditions, fragments were electrophoretically resolved (3 V/cm in 0.8% agarose gels) and transferred to Hybond+ membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom), as described elsewhere (22). Membranes were then probed and developed by using an enhanced chemiluminescence system (Amersham Biosciences), as recommended by the manufacturer.

Several strains did not yield amplicons for one or more loci; however, Southern blot analysis revealed that all 11 gene loci were present in each of the tested strains (Table 3), although atypical restriction patterns were frequently observed (Table 3). Ten distinct classes of restriction (Fig. 2) were identified among the 19 strains showing a Southern blot pattern distinct from UA159 at the *comCD* locus (Table 3). Among the 26 genotypes that were PCR negative for *comCD*, 10 were also

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TABLE 1. Competence genes identified in the genome of the strain UA159 that were analyzed in this study

Gene	GenBank accession no.[a]	Assigned function
mecA	24378754	Negative regulator of genetic competence
luxS	24378961	Putative autoinducer-2 production protein LuxS
comA	24378790	ABC transporter, ATP-binding protein ComA
comB	24378791	ComB, accessory factor for ComA
comF	24378982	Late competence protein, required for DNA uptake
comFa	24378983	Late competence protein
comEA	24378983	DNA uptake protein and related DNA-binding proteins
comEC	24379099	DNA internalization-related competence protein ComEC/Rec2
coiA	24379117	Putative competence protein, transcription factor
ciaH	24379560	Putative histidine kinase sensor CiaH
ciaR	24379561	Putative response regulator CiaR
comC	24380265	Competence stimulating peptide, precursor
comD	24380266	Putative histidine kinase of the competence regulon, ComD
comE	24380267	Putative response regulator of the competence regulon, ComE
comYD	24380327	Putative late competence protein ComYD, exogenous DNA-binding protein
comYC	24380328	Late competence protein, exogenous DNA-binding protein
comYB	24380329	Putative ABC transporter subunit ComYB; part of the DNA transport machinery
comYA	24380330	Putative ABC transporter, ATP-binding protein ComYA; late competence gene
SMU1988c		Putative DNA-binding protein
comX1	24380340	Transcriptional regulator of competence-specific gene
cinA	24380420	Putative competence and damage-inducible protein CinA

[a] Available online (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

PCR negative for *comDE*, suggesting that polymorphism might be involved in at least *comD* or, perhaps, the whole *comCDE* locus. Southern blot analysis of three randomly selected clinical isolates that were PCR positive for *comCD* revealed a UA159-type pattern (data not shown). The lower number of atypical patterns in assays with *comDE* probe, compared to the *comCD* probe (Table 3), might be due to differences in the conservation of the restriction sites used. All of the strains yielded amplicons for *ciaHR*. It is possible that the primer set designed for these genes included highly conserved sequences because ciaHR primers have also generated amplicons for *S. sobrinus* strains, a species closely related to *S. mutans* that is also implicated in dental caries pathogenesis. BLAST analysis of ciaHR primer sequences against the unfinished genome of the strain *S. sobrinus* 6715 (http://www.tigr.org/tdb/mdb/mdbinprogress.html) did not reveal regions of homology that might account for amplification (data not shown). Except for ciaHR primers, all of the others appeared to be *S. mutans* specific, since they did not yield amplicons for the *S. sobrinus* strain 15JP1. In addition, during the course of the present study, a total of 11 strains previously defined as *S. mutans* species were negative in PCR for most other *com* genes analyzed (data not shown). Sequencing analysis of the 16S rRNA gene revealed that these 11 strains were *S. sobrinus* (data not shown) and were thus excluded from the analysis. The reason(s) for the significant differences in the capacities for genetic transformation between *Streptococcus* species and between strains within the same species are not understood. The ability to achieve competence in virulence is unclear. It is hypothesized that incorporation of foreign DNA may improve fitness to environmental stresses, providing competitive advantages (3, 7). There is evidence that some TCS that are involved in competence may regulate multiple virulence factors (10, 12, 24, 27, 28), and their components could be important targets for antibacterial therapy. Thus, it is important to establish the conservation of these systems within the *S. mutans* species.

Sequence comparisons of *comCDE* genes between several species of the genus streptococcus have indicated that interspecies gene replacements may frequently occur between naturally competent streptococci within the *Streptococcus mitis* group (6). Differences in GC content within the *comCDE* locus in comparison with the whole streptococcal genome concur with this idea (7). A locus with homology to *comCDE* (named *silCDE*) was identified in the screening of virulence genes in the *S. pyogenes* genotype JS95 which was isolated from a subject with invasive infection. *S. pyogenes* is a species with a low rate of transformation and causes infections having a wide range of severity (7). Similar to the organization of *comCDE* genes in the genome of *S. mutans* UA159, the *silC*, *silD*, and *silE* genes are flanked by Blp bacteriocin genes and also by a transposable element IS*1562*. The *silCDE* locus was shown to confer competence in the virulent genotype (7), a trait that is not observed in the *S. pyogenes* strain M1 whose genome was sequenced and which does not have *silCDE* genes (7). M1 is defective in transformation, although it contains several *com* genes (4). Its inability to be transformed was attributed to the lack of *comAB* genes (4). In screening 214 strains of *S. pneumoniae*, *comC* and *comA* genes were shown to be widespread (21), and these genes were detected in all of the *S. mutans* strains (Table 3). The upper primer utilized for screening the *comCD* sequence targeted a small intergenic space between open reading frames SMU1914c and SMU1913c (Fig. 1) that encodes homologues of the immunity protein Blpl and a bacteriocin peptide, BlpJ, also identified in the *S. pneumoniae* strain TIGR4.

Because two distinct alleles of *comC* were detected among 42 strains of *S. pneumoniae*, the hypothesis that the combination of incompatible alleles of *comC* (encoding the CSP precursor) and *comD* (the respective receptor) (5) was raised to explain the deficiency in in vitro transformation observed among 50% of the *S. pneumoniae* isolates (19). However, 93% of 60 strains analyzed have shown only two distinct *comC* alleles matched with the respective *comD* alleles (26), arguing against the incompatible allele hypothesis. Thus, other factors may be involved in variations in the competence phenotype. It has been shown in *S. pneumoniae* that the effect of gene inactivation of components of TCS which are involved in virulence is dependent on the strain background (2). Since few *S. mutans* strains were identified as suitably transformable in vitro, most of the genetic studies of molecular mechanisms of virulence have been limited to a few strains, mainly GS5, NG8, and UA159 (and its variant LT11). The distribution and/or diversity of regulatory and structural genes implicated in competence might help to explain differences in competence. However, to our

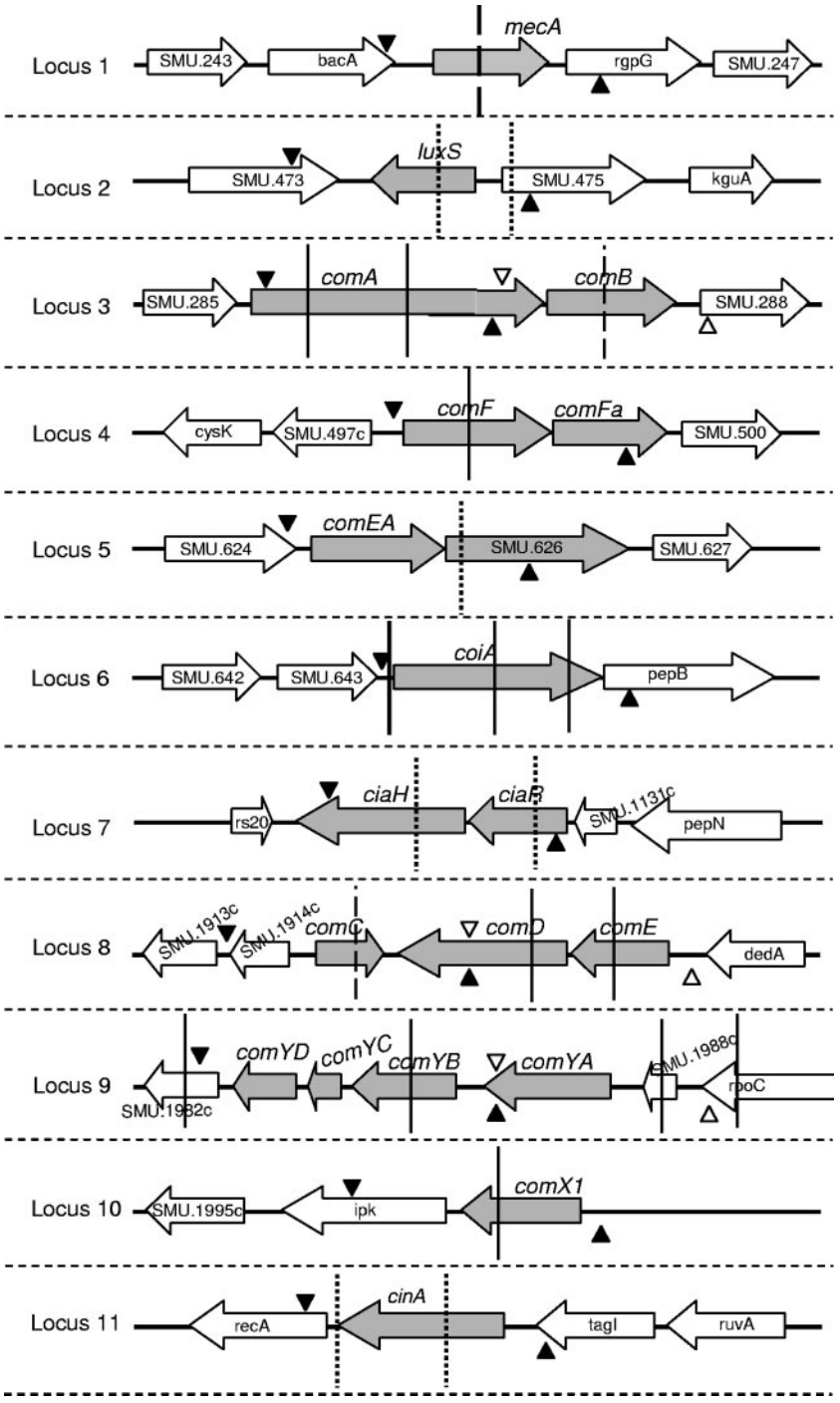

FIG. 1. Genomic organization of the 11 loci identified in the genome of the strain UA159 which contains 21 competence-related genes, as analyzed by PCR and Southern blotting. Shaded arrows indicate the open reading frame of the *com* genes and the direction of transcription. The locations of primers designed for PCR screening are indicated by bullets. Sets of primers designed for each amplicon are differentiated by color (black or white). Vertical lines indicate the restriction sites of the endonucleases selected for the Southern blot analysis within each amplicon: dotted line, HindIII; solid line, HaeIII; and dashed line, HhaI. Purified amplicons identified in PCRs with chromosomal DNA of the strain UA159 were applied as probes for Southern blot analysis.

knowledge, there is no such information describing *S. pneumoniae* or other streptococcal species.

It has been estimated that transformation occurs in only 28% of clinical isolates of *S. mutans* in vitro (25), and it is not known whether this low frequency of transformation has a genetic basis or might be simply due to unsuitable lab conditions. The frequency of transformation is significantly variable among naturally competent strains (e.g., GS5, NG8,

TABLE 2. Oligonucleotides used for PCR screening of genes identified in the 11 *loci* of the *S. mutans* strain UA159 with a known or putative role in the phenotype of competence in *S. mutans*

Locus	Amplicon (expected size in bp)	Primer sequence (forward and reverse)	Annealing temp (°C)	Competence gene(s) within the amplicon
1	MecA (1,083)	5′-GCACGATTCTAACTGGTAGT-3′ 5′-AGGAGTCAAAACAAGTGAAG-3′	52	*mecA*
2	LuxS (1,496)	5′-GATCGTAGTCGAGTTCCTTA-3′ 5′-GCTCATCAATATGCCTAGAT-3′	52	*luxS*
3	ComA (2,900)	5′-GTCATAGCCGTTAACATTCT-3′ 5′-GAGAAATAAGACAGCAAAGC-3′	52	*comA*
	ComAB (1,425)	5′-TTTTAGCTAAGCAAGGTTTC-3′ 5′-CTTTACGTCTGGACTGATTT-3′	52	*comA*, *comB*
4	ComFFa (1,776)	5′-GTGATGGAGAATTTAGAGA-3′ 5′-GTTTTACACTACCATCTTCT-3′	50	*comF*, *comFa*
5	ComEA (1,670)	5′-CTGGAACCCAGAAAAGCATC-3′ 5′-AAAAGGGCAGCTGAATAGCA-3′	50	*comEA*, *SMU.626*
6	CoiA (1,367)	5′-TGAAATCTACTTTAGTCCTT-3′ 5′-AGCTTGTAATTCTTGATAGT-3′	50	*coiA*
7	CiaHR (1,385)	5′-CGAGGTTGAATTTCTGTTAT-3′ 5′-AAATTTTTGATCGTATCTGG-3′	52	*ciaH*, *ciaR*
8	ComCD (1,771)	5′-TATCAGATGAGTTTGTCC-3′ 5′-ATATCACCCAGTATAGTCAG-3′	52	*comC*, *comD*
	ComDE (1,862)	5′-AGAGATTCTATTTGCTGACT-3′ 5′-TATGTAGGAAGAGTTGAACA-3′	50	*comD*, *comE*
9	ComYDA (1,978)	5′-TGACTCAACTGAGTAAGAAT-3′ 5′-GTTTATGCTAGGATGTTAGA-3′	52	*comYD*, *comYC* *comYB*, *comYA*
	ComYA1988 (1,399)	5′-TCTAACATCCTAGCATAAAC-3′ 5′-AGAGGACACAGTAGAAGAGT-3′	50	*comYA*, *SMU1988c*
10	ComX (1,437)	5′-GACAAAGTAGTCGCTAAAGG-3′ 5′-ACATACCCTGCTTTATCTTG-3′	52	*comX*
11	CinA (1,789)	5′-CAATATCAAGAGCCAGACTT-3′ 5′-GTATACCTGCTCAAACGAAT-3′	52	*cinA*

TABLE 3. Structural analysis of 21 competence-related genes organized in 11 loci identified in the chromosome of the strain UA159 in 94 clinical genotypes of *S. mutans*

Amplicon	No. (%) of clinical genotypes			
	PCR screening in 94 genotypes		Southern blot analysis of PCR-negative genotypes	
	PCR positive	PCR negative	Polymorphic pattern	UA159 pattern
MecA	93 (98.9)	1 (1.1)	1 (1.1)	0
LuxS	82 (87.2)	12 (12.8)	8 (8.5)	4 (4.2)
ComA	93 (98.9)	1 (9.3)	0	1 (1.1)
ComAB	88 (93.6)	6 (6.4)	3 (3.2)	3 (3.2)
ComFFa	71 (75.5)	23 (24.4)	12 (12.8)	11 (11.7)
ComEA	89 (94.7)	5 (5.3)	5 (5.3)	0
CoiA	92 (97.9)	2 (2.1)	0	2 (2.1)
CiaHR	94 (100.0)			
ComCD	68 (72.3)	26 (27.7)	19 (20.2)	7 (7.4)
ComDE	73 (88.3)	11 (11.7)	2 (2.1)	9 (9.6)
ComYDB	90 (95.7)	4 (4.3)	1 (1.1)	3 (3.2)
ComYA1988	80 (85.1)	14 (14.9)	0	14 (14.9)
ComX1	84 (89.4)	10 (10.6)	0	10 (10.6)
CinA	90 (95.7)	4 (4.3)	1 (1.1)	3 (3.2)

UA159, LT11, and UA140), and it has been suggested that variation in the genetic background (17) and/or in the expression of late competence genes may be associated with these variations (15). The alternate sigma factor comX1 that appears to regulate several genes involved in late events of competence was detected in all of the strains (Table 3). In contrast to *S. pneumoniae*, in which at least two alleles of *comX1* were identified in the genome (9, 18), our analysis indicated that *S. mutans* genotypes contain only a single copy of *comX1*, as verified for the strain UA159 (1). All other structural and regulatory loci studied here have shown some degree of genetic diversity, although lower in frequency compared to the *comCDE* locus (Table 3). However, a more detailed analysis of these genes should be performed to allow comparisons in degree of conservation, since Southern blot assays were performed here only with PCR-negative strains, and a PCR-positive result does not imply an absence of polymorphisms. Apart from the modest diversity identified, the results indicated that all 11 loci that contain genes with a regulatory or structural role in competence were present in all of the *S. mutans* genotypes analyzed, suggesting that these genes each play a fundamental role in *S.*

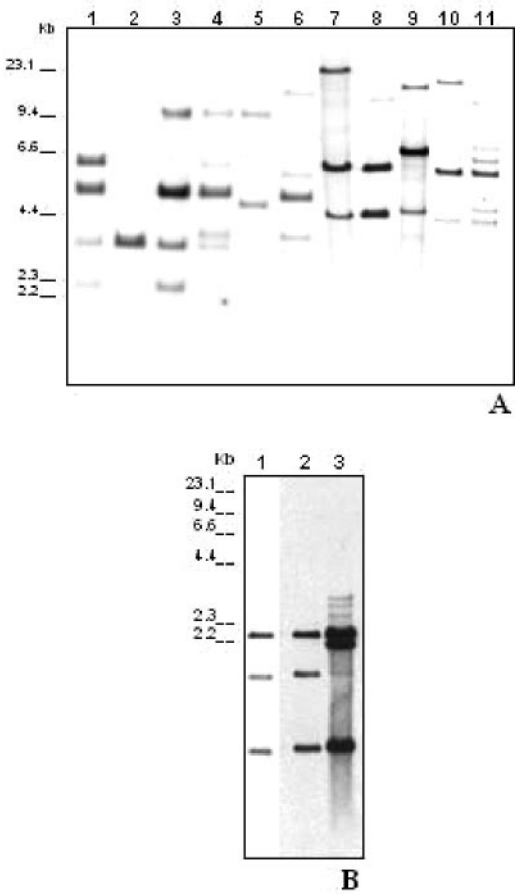

FIG. 2. Southern blot analysis of the *comCDE* locus in UA159 and clinical genotypes that were PCR-negative for comCD (A) and comDE (B) sequences. Lane 1 corresponds to the predicted Southern blot pattern obtained for the control strain UA159. (A) Lanes 2 to 11 correspond to strains representative of each one of the 10 distinct classes of Southern blot identified in 19 strains with atypical Southern blots probed with the comCD amplicon. (B) Lanes 2 and 3 are representative patterns of Southern blots probed with comDE amplicon in the comDE PCR-negative strains. Note that some PCR-negative strains, represented in lane 2, showed the same pattern as strain UA159.

mutans biology. This knowledge may increase the interest in these genes as therapeutic targets. Furthermore, sequencing analysis of the polymorphic strains identified here, along with gene expression studies, might help to explain additional variation in the competence phenotype previously reported within the *S. mutans* species.

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