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Extent of Horizontal Gene Transfer in Evolution of Streptococci of the Salivarius Group▽†

Christine Delorme,[1]* Claire Poyart,[2] S. Dusko Ehrlich,[1] and Pierre Renault[1]

Laboratoire de Génétique Microbienne, Institut National de Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France,[1] and INSERM E44, Institut Cochin, Centre National de Référence des Streptocoques, Service de Bactériologie Hôpital Cochin, Faculté de Médecine Paris 5, 27 rue du faubourg Saint Jacques, 75679 Paris Cedex 14, France[2]

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The phylogenetically closely related species *Streptococcus salivarius* and *Streptococcus vestibularis* are oral bacteria that are considered commensals, although they can also be found in human infections. The relationship between these two species and the relationship between strains isolated from carriers and strains responsible for invasive infections were investigated by multilocus sequence typing and additional sequence analysis. The clustering of several *S. vestibularis* alleles and the extent of genomic divergence at certain loci support the conclusion that *S. salivarius* and *S. vestibularis* are separate species. The level of sequence diversity in *S. salivarius* alleles is generally high, whereas that in *S. vestibularis* alleles is low at certain loci, indicating that the latter species might have evolved recently. Cluster analysis indicated that there has been genetic exchange between *S. salivarius* and *S. vestibularis* at three of the nine loci investigated. Horizontal gene transfer between streptococci belonging to the *S. salivarius* group and other oral streptococci was also detected at several loci. A high level of recombination in *S. salivarius* was revealed by allele index association and split decomposition sequence analyses. Commensal and infection-associated *S. salivarius* strains could not be distinguished by cluster analysis, suggesting that the pathogen isolates are opportunistic. Taken together, our results indicate that there is a high level of gene exchange that contributes to the evolution of two streptococcal species from the human oral cavity.

Streptococcus salivarius and *Streptococcus vestibularis* belong to the salivarius group of viridans streptococci (18). Many of the viridans streptococcal species are part of the normal microbial flora of humans. These commensal species are most prevalent in the oral cavity but also reside in the gastrointestinal and urogenital tracts. Viridans streptococci have emerged as important nosocomial pathogens, and they typically cause significant septicemia when mucosal lesions are present and host defense mechanisms are compromised (36, 53, 57). *S. salivarius* and *S. vestibularis* have been associated with severe human infections, such as, meningitis, endocarditis, and bacteremia (9, 11, 14, 31, 42, 47). Molecular taxonomy based on sequencing and comparison of the 16S rRNA or *sodA* genes demonstrated that these two species are closely related (34, 46).

The population structure of several oral streptococci has been studied previously (7, 19, 26, 38). Genetic typing of commensal or opportunistic pathogens has revealed considerable diversity, sometimes even within a single host. Data obtained in a *Streptococcus mitis* population analysis support the concept that the strains are transient, and this species appears to be maintained by clonal replacement of evolving strains rather than by stable strains (19, 26). Almost all isolates of *Streptococcus mutans* obtained either from 30 individuals or from a small group of families displayed distinctive restriction fragment length polymorphism or restriction endonuclease analysis patterns (7, 38). Fitzsimmons et al. suggested that the high degree of diversity observed in several mucosal bacteria may be a mechanism for avoiding immune elimination (19). *S. mitis* and *Streptococcus oralis* strains isolated from the blood of neutropenic cancer patients were also highly diverse, as they all had distinct fingerprint patterns (57). The population structure and genetic diversity of *S. salivarius* and *S. vestibularis* have been not extensively investigated, in spite of the fact that *S. salivarius* is the predominant oral species, especially during mouth colonization, and can be associated with caries (2, 35, 43).

Multilocus sequence typing (MLST), a method based on the nucleotide sequences of ~500-bp internal fragments of multiple (usually about seven) housekeeping genes, has been widely used to study global epidemiology and bacterial population structure (15). MLST can reveal highly clonal populations, as well as freely recombining populations, and the latter are exemplified by *Streptococcus uberis* or *Streptococcus pneumoniae* populations (8, 16). Several reports have indicated that most streptococcal species have a highly recombinational population structure (33, 37, 58). The genetic relationships of group B, C, G, and A streptococcal isolates from asymptomatic carriers and from human infections were investigated by phylogenetic analysis of MLST data (3, 32, 33). Here we used the MLST method to investigate the genetic relationship between strains of *S. salivarius* and strains of *S. vestibularis*. Below we describe an analysis of data obtained from 27 *S. salivarius* strains and 9 *S. vestibularis* strains recovered from patients with septicemia or from the oral cavities of healthy individuals. Our results document the population structure of *S. salivarius*, the relationship between the two species, and the extent of gene exchange in the evolution of these oral streptococci.

* Corresponding author. Mailing address: Laboratoire de Génétique Microbienne, Institut National de Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France. Phone: (33) 1 34 65 25 26. Fax: (33) 1 34 65 25 21. E-mail: christine.delorme@jouy.inra.fr.

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TABLE 1. Strains and MLST data

Strain	ST	Allele									Source[b]	Origin	Yr
		glck	*ddlA*	*pepO*	*ilvC*	*thrS*	*pyrE*	*dnaE*	*sodA*	*tkt*[a]			
S. salivarius strains													
CIP53.158	1	2	10	7	13	4	15	14	13	NA	NV	Ithaca, NY	1954
CIP55.126	2	2	11	7	13	4	15	14	13	NA	NV	France	1955
CIP102505	3	7	6	1	4	13	12	20	2	NA	NV	New York, NY	1971
JIM8222	4	16	26	13	16	17	22	13	8	NA	Oral cavity	Germany	2000
JIM8223	5	9	7	18	10	4	16	18	20	NA	Oral cavity	Germany	2000
JIM8224	6	14	30	16	10	15	19	12	5	NA	Oral cavity	Germany	2000
JIM8221	7	4	3	3	4	14	12	17	14	NA	Oral cavity	Germany	2000
CIP102503^T	8	3	7	1	8	1	13	17	12	NA	Human blood	New York, NY	1950
CIP104994	9	6	6	4	5	5	11	20	14	NA	Human blood	Tourcoing, France	1996
LMG13104	10	1	15	17	14	2	14	22	10	NA	NV	Munchen, Germany	1983
LMG13106	11	1	17	22	15	4	3	24	2	NA	NV	Hospital, London, United Kingdom	1983
LMG13108	12	15	25	7	11	8	24	16	6	NA	NV	Hospital, London, United Kingdom	1983
LMG13109	13	15	27	20	9	12	21	7	2	NA	NV	Hospital, London, United Kingdom K	1983
LMG14652	14	5	14	3	5	5	15	21	14	NA	Human blood	Ostersund, Sweden	1993
JIM8421	15	15	23	11	14	11	2	23	11	NA	Breast milk	Finland	2001
JIM8775	16	2	16	6	12	4	15	17	7	NA	Oral cavity 1	France	2004
JIM8777	18	1	9	17	6	7	4	19	10	NA	Oral cavity 1	France	2004
JIM8776	17	2	12	12	7	6	10	15	9	NA	Oral cavity 1	France	2004
JIM8774	17	2	12	12	7	6	10	15	9	NA	Oral cavity 2	France	2004
JIM8773	21	17	4	21	9	1	20	9	4	NA	Oral cavity 2	France	2004
JIM8771	19	16	8	6	14	9	23	8	1	NA	Oral cavity 2	France	2004
JIM8772	20	8	28	2	8	10	25	17	7	NA	Oral cavity 2	France	2004
CCHSS1	22	16	29	14	17	20	18	11	3	NA	Human blood	Hospital Cochin, France	2003
CCHSS2	23	14	1	8	10	16	19	10	2	NA	Human blood	Cochin Hospital, France	2001
CCHSS3	24	5	13	19	5	4	1	6	14	NA	Human blood	Cochin Hospital, France	2002
CCHSS4	25	4	24	5	5	5	11	20	2	NA	human blood	Cochin Hospital, France	2001
CCHSS7	26	15	2	16	12	6	17	17	1	NA	human blood	Cochin Hospital, France	2004
S. vestibularis strains													
CIP103363^T	1	12	19	10	3	3	9	2	16	5	Oral cavity	Hospital, London, United Kingdom	1987
LMG17854	2	11	20	9	1	18	7	4	17	4	Vestibular mucosa	Malmö, Sweden	1966
LMG14646	2	11	20	9	1	18	7	4	17	4	Vestibular mucosa	Malmö, Sweden	1966
LMG14645	3	13	21	9	1	19	7	4	15	4	Vestibular mucosa	Malmö, Sweden	1966
LMG14647	4	10	18	9	2	18	5	1	15	3	Vestibular mucosa	Malmö, Sweden	1966
LMG17855	4	10	18	9	2	18	5	1	15	3	Vestibular mucosa	Malmö, Sweden	1966
LMG17856	4	10	18	9	2	18	5	1	15	3	Human dental plaque	Malmö, Sweden	1966
CCHSV5	5	10	22	15	1	21	8	5	18	1	Human blood	Cochin Hospital, France	2002
CCHSV6	6	10	5	10	1	3	6	3	19	2	Human blood	Cochin Hospital, France	2004

[a] NA, not amplified.
[b] NV, not available.

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MATERIALS AND METHODS

Bacterial strains. *S. salivarius* (n = 27) and *S. vestibularis* (n = 9) strains are listed in Table 1. These strains were isolated in different countries and from different sources over a 50-year period (1954 to 2004) and were obtained from the Institut Pasteur Collection (CIP), the Belgium Coordinated Collections of Microorganisms (LMG), Paris Cochin Hospital (CCH), and our laboratory collection (JIM). Strains were obtained from the oral cavity (n = 18), human blood (n = 10), and breast milk (n = 1). Commensal strains were isolated from different oral cavities; oral cavities 1 and 2 were the oral cavities of 3-year-old twin girls.

Strains were grown overnight at 37°C on M17 broth medium containing glucose or lactose at final concentration of 1% in an anaerobic atmosphere. Samples were collected from oral cavities by scraping the tongue surface with sterile wooden tongue depressors that were used directly for isolation on M17 medium plates containing glucose. Both genetic identification and phenotypic identification were performed for all the strains. Phenotypic identification was performed with the rapid ID 32 Strep system (bioMérieux) or using a focused scheme for assessing hydrolysis of urea and acid production in lactose-, raffinose-, sorbitol-, or inulin-containing media. Hydrolysis of urea and a failure to ferment raffinose and inulin are characteristics of all *S. vestibularis* strains tested (10). Genetic identification at the species level was carried out by sequencing 16S rRNA and the *sodA* gene (46).

Multilocus sequence typing. We searched for candidate MLST loci among housekeeping genes which were used previously for MLST of other gram-positive bacteria and which could be amplified with degenerate primers (*ddlA*, *tkt*, *glcK*, *pyrE*, and *sodA*) or whose sequences were available in a database for closely related species (*ilvC*, *pepO*, *thrS*, and *dnaE*). Furthermore, we checked to make sure that the genes were usually found in distant locations in previously described streptococcal genomes, especially the genome of the closely related species *Streptococcus thermophilus*. The nucleotide sequences of internal fragments of the following nine genes that were selected were determined: *ilvC* (encoding ketol-acid reductoisomerase), *ddlA* (encoding D-alanine D-alanine ligase), *glcK* (encoding glucose kinase), *pepO* (encoding endopeptidase), *thrS* (encoding threonyl-tRNA synthetase), *tkt* (encoding transketolase), *pyrE* (encoding orotate phosphoribosyltransferase), *dnaE* (encoding DNA polymerase III), and *sodA* (superoxide dismutase). For the *sodA* gene, we used primers and an amplification procedure described previously (46). Primers used in this study are listed in

TABLE 2. Oligonucleotide primers for *S. salivarius* and *S. vestibularis*

Locus	Accession no.	Primer function	Primer	Sequence (5′–3′)
ilvC	AF220670	MLST	ilvC-up	GATCAGGTCACGATGTTAT
		MLST	ilvC-dn	GGTGCATATCCAGCTTCAGT
ddlA	gbU69167	MLST	ddlA-up	TCAAGTGTGGCTATGGA
		MLST	ddlA-dn	GTAGATGGCTCCATCCTC
glcK	AF442552	MLST	glcK-up	TGGGCAGAAACTCAAGA
		MLST	glcK-dn	AACACCACCACCGATAAC
pyrE	CP000024	MLST	pyrE-dn	CGCTTTACGGAGGAACAT
		MLST	pyrE-up	GTCCGTCTGCAGTGATGT
thrS	CP000024	MLST	thrS-up	ATCACTGAAGATGGAAGC
		MLST	thrS-dn	CCAAGTTTACGGTGGTCA
dnaE	CP000024	MLST	dnaE-up	GGACTGGGAGCCTGGGAT
		MLST	dnaE-dn	ACTCCCTGCAGCAGACCC
pepO	AJ249396	MLST	pepOup	AACTCTACCACCCTTATGA
		MLST	pepOdo	GGTTTGTTCCACTTGCTCCAG
tkt	CP000024	MLST (*S. vestibularis*)	tkt-up	GCAGCACCAATGGGTTAC
		MLST (*S. vestibularis*)	tkt-dn	CCAAGTTTACGGTGGTCA
			tkt-up2	CTGGCCACCCTGGTGTGG
		.	tkt-dn2	GGAGCACCGTGAACACCG
			tkt-up3	AATGCTATTCGTTTTCTAGG
			tkt-dn3	TTGAAATCAGCATATACTT
			tkt-up4	CAGGTCATGGTTCAATGCT
			tkt-dn4	TGAATTACGAGTTGCTTG
			tkt-dn5	TTGATATCATTTGAATC
			tkt-up6	ATGGGTGCAGCACCAATGG
			tkt-dn6	ACCAATGACTGTCTTAAC
tkt region		Large region	tkt-dn7	CTTAGAACCCCATTGACGG
		Large region	tkt-up8	GGTGTACGTGAGTTTGCG
		Large region	tkt-salup	CTTGGCATTGCTTGACTTCG
		Large region	tkt-saldn	CCTTCGATTGAGATTAGTAAC
		Large region	tkt-saldn8	CAAAGGCAGCCTTGTATTCC
		Large region	tkt-saldo9	CAGAACCAGTCGCAATGA
		Large region	tkt-vesup	CAAGATTATCTTGTCATCG
		Large region	tkt-vesdo	GGCTGGAAAGTCCTTGCATAC
		Large region	tkt-vesup9	GGCAGCAAGTCTACCATGG
		Large region	tkt-vesdo9	GCTTGATACCATGCAGCC-
ilvC region		Large region	ilvB-up3	GTGGACAGCTCAGTATTATC
		Large region	ilvC-dn3	GCTTCTGCTACTGTGTAAG
		Large region	ilvC-up5	CTGAAGAAGATTTGTTTGGTG
		Large region	tyrS-do4	GGTAACATGACAGCAGGTAC
pepO region		Large region	pepO-up6	GCAGGGTGCGTGCTCTTG
		Large region	pepO-up4	ACGCTTACAAGATGATTT
		Large region	pepO-dn6	CTTCTGGAACGATGATTTTATC
		Large region	pepO-dn4	TCATAAGGGTGGTAGAGTT
		Large region	pepO-dn5	TACCAAATAATAACACGATC
		Large region	pepO-dn7	TAGTCATATGAAACTCCT
		Large region	pepO-up5	CTGGAGCAAGTGGAACAAACC
		Large region	pepO-up7	GAAATTTCAGTTGCTCATAG

Table 2. One bacterial colony, freshly grown on an agar plate, was suspended in 50 μl of TES (10 mM Tris-HCl, 1 mM EDTA, 25% sucrose), and DNA was extracted by lysis in a thermocycler at 95°C for 10 min and at 4°C for 15 min. The DNA obtained was used immediately for PCR amplification performed with the GeneAmp 9700 PCR system (Perkin-Elmer) by using the following cycling parameters: 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. Amplified products (459 to 527 bp of internal gene fragments) were examined on a 1.5% agarose gel. They were used for direct sequencing by a fluorescent sequencing procedure with the same PCR primers that were used for the initial PCR amplification. The sequence of each fragment on both strands was determined with an Applied Biosystems 370A DNA sequencer by using a Bigdye Terminator sequencing kit according the manufacturer's protocol (Perkin-Elmer). To ensure the accuracy of the sequence, amplicons obtained from at least two bacterial colonies were used for sequence determination.

PCR amplification of *ilvC*, *pepO*, *pyrE*, and *tkt* genes for sequencing. Primer pairs for the *tkt* locus did not amplify the DNA fragments of all *S. salivarius* isolates. Therefore, alternative sets of primers were designed using sequence information available in public databases. The primers listed in Table 2 (*tkt*-up2, *tkt*-dn2, *tkt*-up3, *tkt*-dn3, *tkt*-up4, *tkt*-dn4, *tkt*-dn5, *tkt*-up6, and *tkt*-dn6) were used in all possible up and dn combinations. The large region around the *ilvC*, *pepO*, and *tkt* genes was sequenced by primer walking, using primers designed on the basis of the regions sequenced. For amplification of the region containing the *ilvB*, *ilvN*, *ilvC*, and *tyrS* genes, we used *ilvB*-up3, *ilvC*-dn3, *ilvC*-up5, and *tyrS*-do4 (Table 2). For amplification of the region containing the *dexS* and *pepO* genes, we used *pepO*-up6, *pepO*-up4, *pepO*-dn6, *pepO*-dn4, *pepO*-dn5, *pepO*-dn7, *pepO*-up5, and *pepO*-up7 (Table 2). For amplification of the region containing the *tkt* gene, we used *tkt*-dn7 and *tkt*-up8 for both species, *tkt*-salup, *tkt*-saldn, *tkt*-saldn8, and *tkt*-saldo9 for *S. salivarius*, and *tkt*-vesup, *tkt*-vesdo, *tkt*-vesup9, and *tkt*-vesdo9 for *S. vestibularis* (Table 2).

For *pyrE* sequence alignment, the nucleotide sequence of the mobile element found in allele 11 (*S. salivarius*) was not taken into account.

Data treatment and statistical analysis. For each locus, all the sequences were compared, and arbitrary allele numbers were assigned to the different sequences. The combination of alleles at each locus defined an allelic profile or sequence type (ST) for a strain. Strains with the same allelic profile were assigned to the same ST. The STs were identified by arbitrary numbers.

The number of polymorphic nucleotide sites and the maximal and average levels of nucleotide divergence of alleles (expressed as percentages) at a given

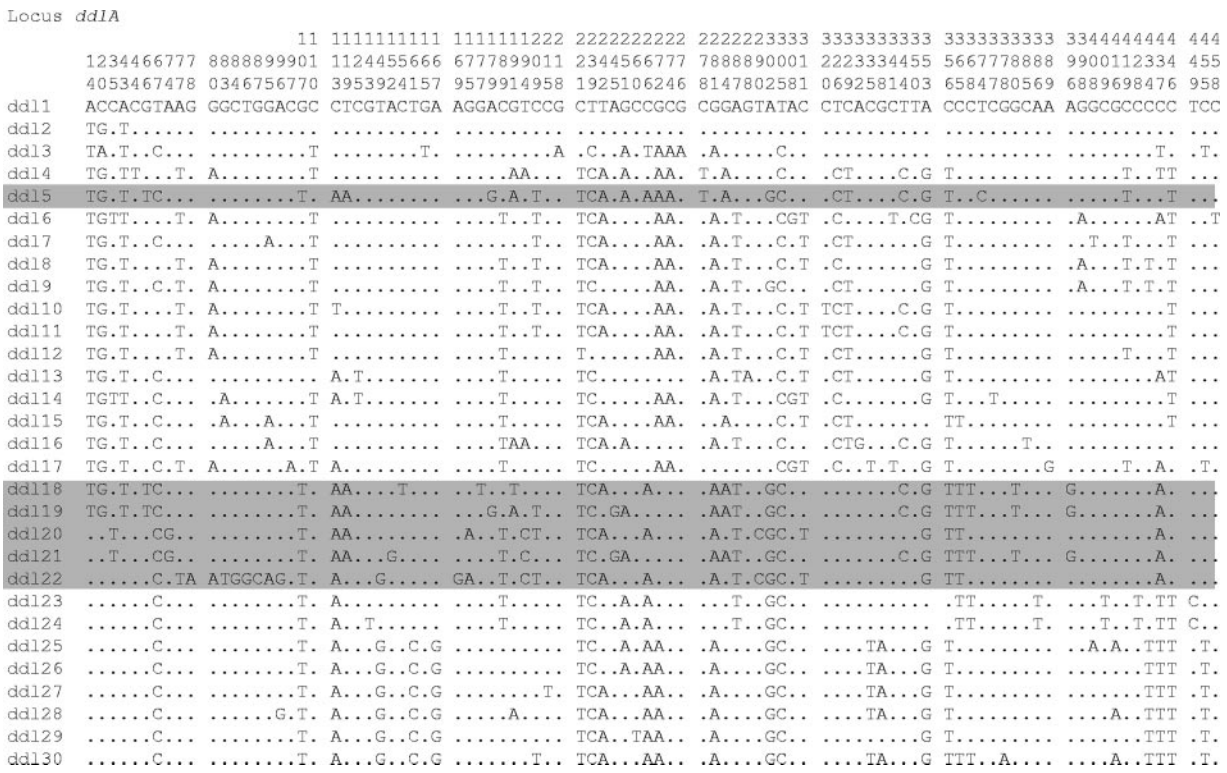

FIG. 1. Polymorphic nucleotide sites of *ddlA* alleles of *S. salivarius* and *S. vestibularis*: variable sites in each allele of the *ddlA* locus from the 27 *S. salivarius* strains and 9 *S. vestibularis* strains. The site numbers at the top are in vertical format, and *S. vestibularis* alleles are shaded.

locus were calculated using the MEGA software (version 3; http://www.megasoftware.net) (39). Phylogenetic analyses of the nucleotide sequences of each housekeeping gene separately and the concatenated sequence containing *ddlA*, *thrS*, *pyrE*, *dnaE*, and *sodA* were performed using the neighbor-joining (NJ) method and the same software. Because of their extensive nucleotide diversity, the *glcK*, *ilvC*, and *pepO* sequences could not be used in the MLST scheme, so we removed them from the concatenated sequence. A Kimura two-parameter distance model was used to estimate distances for nucleotide sequences. To determine the significance of the groups observed in trees constructed by the NJ method, a bootstrap analysis with 1,000 replicates was performed.

The split decomposition method was used to assess the degree of tree-like structure for alleles found for each locus (30). The sequence alignments were converted to NEXUS files, and the split decomposition analysis was performed with the SPLITSTREE 3.1 program (http://bibiserv.techfak.uni-bielefeld.de/splits/).

A statistical analysis based on the d_N/d_S ratio (41), which was the ratio of the number of substitutions that changed the amino acid sequence (d_N is number of nonsynonymous substitutions per nonsynonymous site) to the number of silent substitutions (d_S is the number of synonymous substitutions per synonymous site), was performed by using methods described with the START program (K. Jolley; http://mlst.zoo.ox.ac.uk/links/START). The standardized index of association (I^S_A) was calculated as described by Haubold and Hudson (24). In our study, the set of *S. salivarius* isolates analyzed for linkage equilibrium did not contain CIP55.126 and JIM8774, which are considered to be very closely related to CIP53.128 and JIM8776, respectively.

Nucleotide sequence accession numbers. The sequences of all alleles have been deposited in the GenBank database under accession numbers DQ460524 to DQ460540 (*ilvC* fragment), DQ460609 to DQ460638 (*ddlA* fragment), DQ460507 to DQ460523 (*glcK* fragment), DQ460541 to DQ460562 (*pepO* fragment), DQ460588 to DQ460608 (*thrS* fragment), DQ460478 to DQ460482 (*tkt* fragment), DQ460563 to DQ460587 (*pyrE* fragment), DQ460483 to DQ460506 (*dnaE* fragment), and EF054776 to EF054795 (*sodA* fragment). The sequences of large regions have been deposited in the GenBank database under accession numbers DQ46995 (*dexS* and *pepO* of *S. salivarius* LMG13109), DQ464996 (*dexS* and *pepO* of *S. vestibularis* LMG14645), DQ464998 (*ilvB*, *ilvN*, *ilvC*, and *tyrS* of *S. salivarius* LMG13109), DQ464994 (*ilvB*, *ilvN*, *ilvC*, and *tyrS* of *S. vestibularis* LMG14645), DQ464997 (*tkt* and $tRNA^{Thr}$ of *S. salivarius* LMG13109), and DQ464993 (*orf1*, *tkt*, and *trkA2* of *S. vestibularis* LMG14645).

RESULTS

Development of an MLST scheme for *S. salivarius* and *S. vestibularis*. Twenty-seven *S. salivarius* strains and nine *S. vestibularis* strains isolated from different sources (oral cavity, breast milk, and human blood) and in different countries over a 50-year period were collected (Table 1). Misclassification due to the natural intraspecies phenotypic variability and the high level of 16S rRNA sequence identity (99%) between *S. salivarius* and *S. vestibularis* was corrected by characterization of the *sodA* gene sequence. The type strains of *S. salivarius* (CIP102503 [= NCTC8618]) and *S. vestibularis* (CIP103363 [= NCTC 12166]) were used as standards. The nucleotide sequences of 404- to 527-bp internal fragments were determined for eight and nine loci in *S. salivarius* and *S. vestibularis*, respectively, as *tkt* could not be amplified for *S. salivarius* with the first set of primers. The polymorphic sites for the *ddlA* alleles are shown in Fig. 1 and for the eight other loci in Fig. S1 in the supplemental material. The average levels of sequence diversity for the *S. salivarius* and *S. vestibularis* alleles were 5.8% and 2%, respectively.

In the *S. salivarius* population, a high number of unique alleles was found for each gene, and there was no particularly predominant allele. The number of alleles per locus for the 27 *S. salivarius* strains ranged from 13 (*glcK*) to 24 (*ddlA*). The highest level of nucleotide sequence divergence for a locus ranged from 4.9% (*sodA*) to 45.3% (*pepO*) (Table 3). The

TABLE 3. Genetic diversity at *S. salivarius* and *S. vestibularis* loci

Locus	Length of sequence (bp)	No. of alleles		No. of variable sites		Maximal % divergence		
		S. salivarius (27 isolates)	*S. vestibularis* (9 isolates)	*S. salivarius* (27 isolates)	*S. vestibularis* (9 isolates)	Within *S. salivarius*	Within *S. vestibularis*	Between *S. salivarius* and *S. vestibularis*
glcK	470	13	4	65	4	11.9	0.9	11.7
pepO	460	19	3	193	138	45.3	38.2	41.2
ilvC	492	14	3	82	3	15.5	0.6	16
sodA	404	15	5	29	3	4.9	0.7	11.6
thrS	497	17	4	60	27	6.1	4	6.8
pyrE	519	20	5	69	3	7.3	0.6	7.3
dnaE	480	19	5	76	34	6.7	6.3	11.1
ddlA	459	24	6	75	50	8.1	8.8	9.3
tkt[a]	527		5		4		0.6	

[a] The *tkt* locus was amplified only in *S. vestibularis* isolates.

d_N/d_S ratio was calculated as a measure of the degree of selection in the population. For *S. salivarius*, the d_N/d_S ratios for *glcK*, *pyrE*, *ilvC*, *sodA*, and *dnaE* were <0.25. In contrast, the d_N/d_S ratio for the *thrS*, *ddlA*, and *pepO* loci were >1, indicating that there was a lower degree of selection against amino acid change (32).

Table 1 summarizes the allelic profiles of the 27 *S. salivarius* strains used in this study. Each unique combination of allele numbers represents one allelic profile or ST. For *S. salivarius*, 26 different allelic profiles were found (ST1 to ST26), which allowed discrimination of all but two strains in our collection (ST17) (Table 1). Most STs differed at at least three of seven loci; the exceptions were ST1 and ST2, which differed at only one locus (Table 1). ST1 and ST2 were represented by two strains from the Institut Pasteur Collection (CIP53.158 and CIP55.126) and differed by only one nucleotide in the *ddlA* allele (Fig. 1 and Table 1). This is in sharp contrast to the high levels of variability observed for most other isolates and suggests that CIP53.158 and CIP55.126 originated from the same strain. The two ST17 strains, JIM8776 and JIM8774, are probably independent isolates of the same strain originating from the oral cavities of twin girls (Table 1). However, analysis of multiple isolates from the same two oral cavities revealed three different STs in one oral cavity and four different STs in the other, showing that *S. salivarius* isolates from the same individual are genetically heterogeneous. For the commensal and disease-associated *S. salivarius* isolates, the concatenated *ddlA*, *thrS*, *pyrE*, *dnaE*, and *sodA* sequences (total length, 2,359 bp) did not allow us to clearly distinguish the two classes, as judged from the tree shown in Fig. 2.

For the set of nine *S. vestibularis* strains, the maximal level of nucleotide sequence divergence ranged from 0.6% (*pyrE*) to 38.2% (*pepO*) (Table 3), and six different allelic profiles were found, (ST1 to ST6) (Table 1). Two and three strains belonged to ST2 and ST4, respectively. All of these strains were isolated from vestibular mucosa at the same time and place (1966 in Malmö, Sweden). The strains with the same ST might correspond to multiple isolates of the same strains (Table 1). Because of the low number of independent isolates in our set, we could not use *S. vestibularis* in the MLST scheme.

Evidence of intraspecific recombination in *S. salivarius* obtained by statistical analysis. Two types of statistical analysis were used to characterize intragenic recombination at *S. salivarius* loci analyzed by MLST.

(i) Standardized index of association. The index of association has been widely used to analyze the degree of linkage disequilibrium between alleles in MLST and multilocus enzyme electrophoresis data (49). Haubold and Hudson described the standardized index of association, which does not

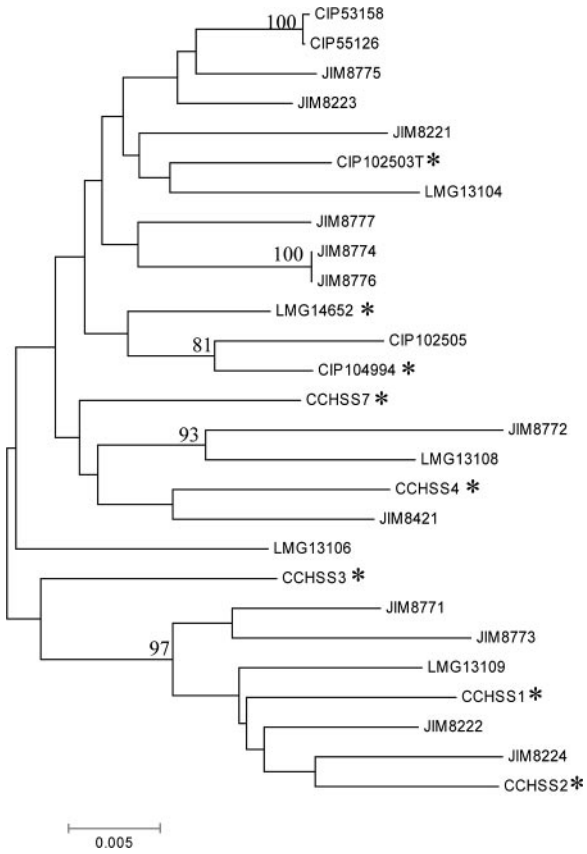

FIG. 2. Phylogenetic tree based on concatenated sequences of five housekeeping genes (*ddlA*, *thrS*, *pyrE*, *dnaE*, and *sodA*). The tree was constructed using the neighbor-joining method. Bootstrap values that are ≥80% are indicated at nodes. Scale bar = 0.005 nucleotide substitution per site. Disease-related isolates are indicated by an asterisk.

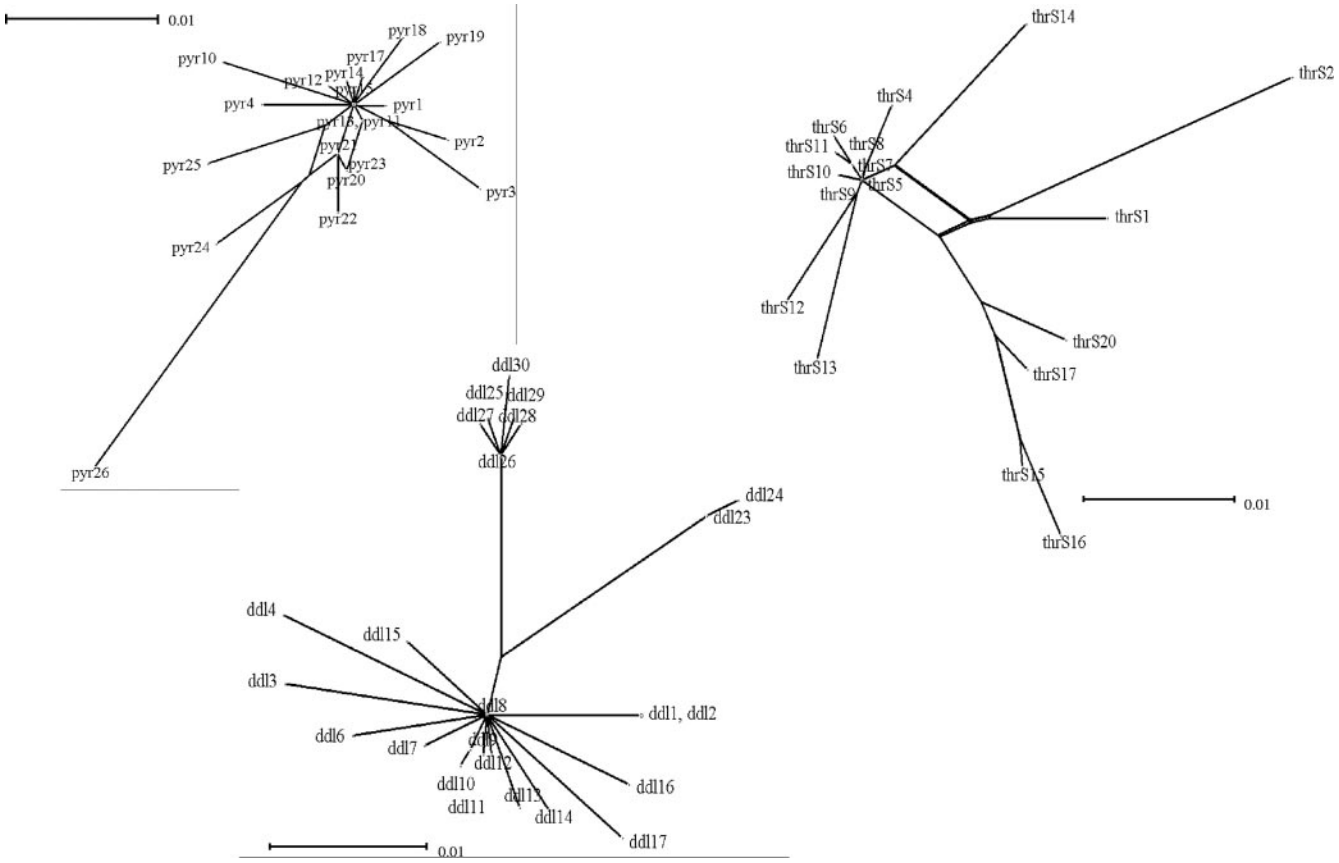

FIG. 3. Split decomposition analysis of alleles present in 27 *S. salivarius* strains: split graphs for *thrS*, *pyrE*, and *ddlA* alleles. In some graphs several alleles are connected to each other by multiple pathways, forming an interconnected network and suggesting that there were recombination events. The numbers are allele numbers. Split graphs for *ilvC*, *pepO*, *glcK*, *dnaE*, and *sodA* alleles are shown in Fig. S2 in the supplemental material.

depend on the number of loci analyzed (24). An I^S_A value of zero is expected for alleles in linkage equilibrium, indicating that alleles are distributed independent of each other because of free recombination (24). The I^S_A value calculated for our *S. salivarius* data was close to zero (0.057), suggesting that there was free recombination and thus a high level of gene exchange.

(ii) Split decomposition analysis. Evidence of recombination was obtained by split decomposition analysis using the SPLITSTREE 3.1 program. The algorithm used in this software is able to display conflicting results in the phylogenetic descent of sequences. A tree-like structure is created when the descent is clonal, but an interconnecting network-like structure appears when recombination plays a role in the evolutionary history of the population analyzed (30, 52). The results obtained with unique *S. salivarius* alleles are shown in Fig. 3 and in Fig. S2 in the supplemental material. All but one split graph have network-like structures consistent with a recombinational population structure. The sole exception is the *ddlA* split graph, which is closer to a bush-like structure.

Clustering *S. salivarius* and *S. vestibularis* allele trees. The relationship between *S. salivarius* and *S. vestibularis* strains was analyzed further by using the nucleotide sequences of the alleles at each MLST locus. Allele trees that included all *S. salivarius* and *S. vestibularis* alleles were constructed for each of the seven loci by using the NJ method (48) (Fig. 4). On the one hand, as expected for a separate species, *S. vestibularis* alleles are clustered together in the *glcK*, *pyrE*, *dnaE*, *sodA*, and *ilvC* trees, and this clustering is supported by bootstrap values of 100%. On the other hand, the *ddlA*, *pepO*, and *thrS* allele trees do not allow discrimination between *S. vestibularis* and *S. salivarius* alleles (Fig. 4). For example, in the case of *pepO*, the *S. vestibularis pepO15* allele (see Fig. S1 in the supplemental material) clearly clusters with *S. salivarius* alleles such as *pepO14* (5.2% divergence), while it exhibits more than 38% divergence with other *S. vestibularis* alleles. In the same way, the *ddlA* and *thrS* alleles of both species are mixed in different branches (Fig. 1; see Fig. S1 in the supplemental material).

Origin of *S. salivarius* and *S. vestibularis* genes. In *S. salivarius*, the maximal level of divergence of nucleotide sequences ranged from less than 10% (4.9 to 8.1%) for five loci to more than 10% for three loci (11.9 to 43.5%; *pepO*, *ilvC*, and *glcK*) (Table 3). In the same way, in *S. vestibularis* the maximal level of divergence of nucleotide sequences was 38.2% for the *pepO* locus (Table 3). Interestingly, the *pepO*, *ilvC*, and *glcK* trees were each divided into two clusters strongly supported by a bootstrap value of 100% (Fig. 4). None of the clusters corresponded to known features, such as species or origin (commensal versus pathogen or country). For each locus, the average levels of divergence were similar within the cluster, whereas they were three- to fourfold higher between the clus-

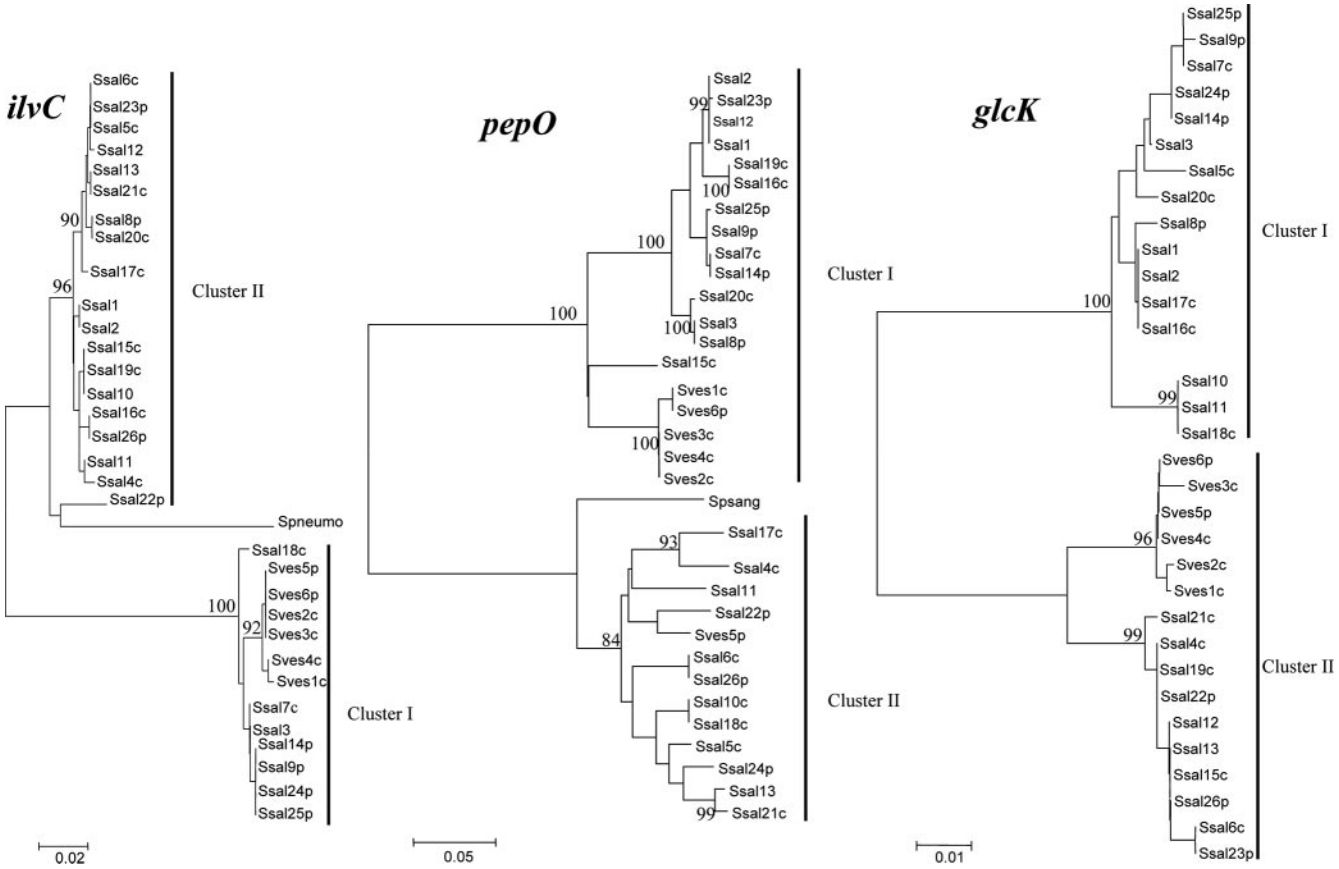

FIG. 4. Phylogenetic relationships among 27 *S. salivarius* strains and 9 *S. vestibularis* strains. Trees are shown for *glcK*, *ilvC*, and *pepO* loci. Ssal, *S. salivarius*; Sves, *S. vestibularis*; p, pathogen; c, commensal; Spneumo, *S. pneumoniae*; Spsang, *S. parasanguinis.* The strain numbers correspond to ST numbers shown in Table 1. The trees were constructed by using the neighbor-joining method. Clusters and bootstrap values that are ≥80% are indicated. The scale bars indicate the number of nucleotide substitutions per site.

ters (Table 4). Interestingly, the *pepO* cluster II sequences were more closely related to the *Streptococcus parasanguinis pepO* sequence (GenBank accession no. AF116532) than to cluster I sequences (Fig. 4). In the same way, *ilvC* cluster II sequences were more closely related to *S. thermophilus* (GenBank accession no. AE008420 and AE007356) and *S. pneumoniae* (GenBank accession no. AF220670) *ilvC* sequences than to cluster I sequences (Fig. 4).

The levels of divergence for *pepO* and *ilvC* regions in strains having alleles that belong to different clusters are summarized in Fig. 5. The 1,783-bp cluster II *pepO* sequence exhibited 10.5% divergence from *S. parasanguinis pepO*, compared with 30% divergence from the cluster I *pepO* allele (Fig. 5a). Similarly, the 725-bp cluster II *ilvC* sequence exhibited 5.1% and 10.2% divergence from *S. thermophilus* and *S. pneumoniae ilvC* sequences, respectively, compared with 16.5% divergence from cluster I *ilvC* alleles (Fig. 5b). These results suggest that there was horizontal gene transfer (HGT) of the *pepO* and *ilvC* alleles from different streptococcal species. A similar process seems to take place at the *glcK* locus, as the alleles are split in two well-supported clusters. However, neither of the clusters appears to be closely related to known sequences (Table 4 and Fig. 4), suggesting that there was HGT from an uncharacterized streptococcal species.

TABLE 4. Genetic diversity of clusters at the *glcK*, *ilvC*, and *pepO* loci

Locus	No. of alleles		Maximal % divergence		
	Cluster I	Cluster II	Within cluster I	Within cluster II	Between cluster I and cluster II
glcK	7	9	3.9	2.6	11.9
ilvC	11	6	2.1	4.2	16
pepO	11	11	13.2	12.9	45.3

Genomic features that distinguish *S. salivarius* and *S. vestibularis*. The maximal level of nucleotide divergence was high (4.9 to 45%) at all loci in *S. salivarius*. In contrast, several *S. vestibularis* loci (*glcK*, *ilvC*, *tkt*, *sodA*, and *pyrE*) diverged very little (maximal divergence, <0.9% [Table 3]). These differences in the levels of allele divergence indicate that these loci evolved differently and might be used to develop rapid test to differentiate the two species.

Strikingly, the *tkt* loci were significantly different in *S. salivarius* and *S. vestibularis* as the *S. salivarius* gene could not be amplified with several oligonucleotide pairs. Successful amplification of an internal fragment was achieved only with oligonucleotides *tkt*-up6 and *tkt*-dn6, and the fragment was se-

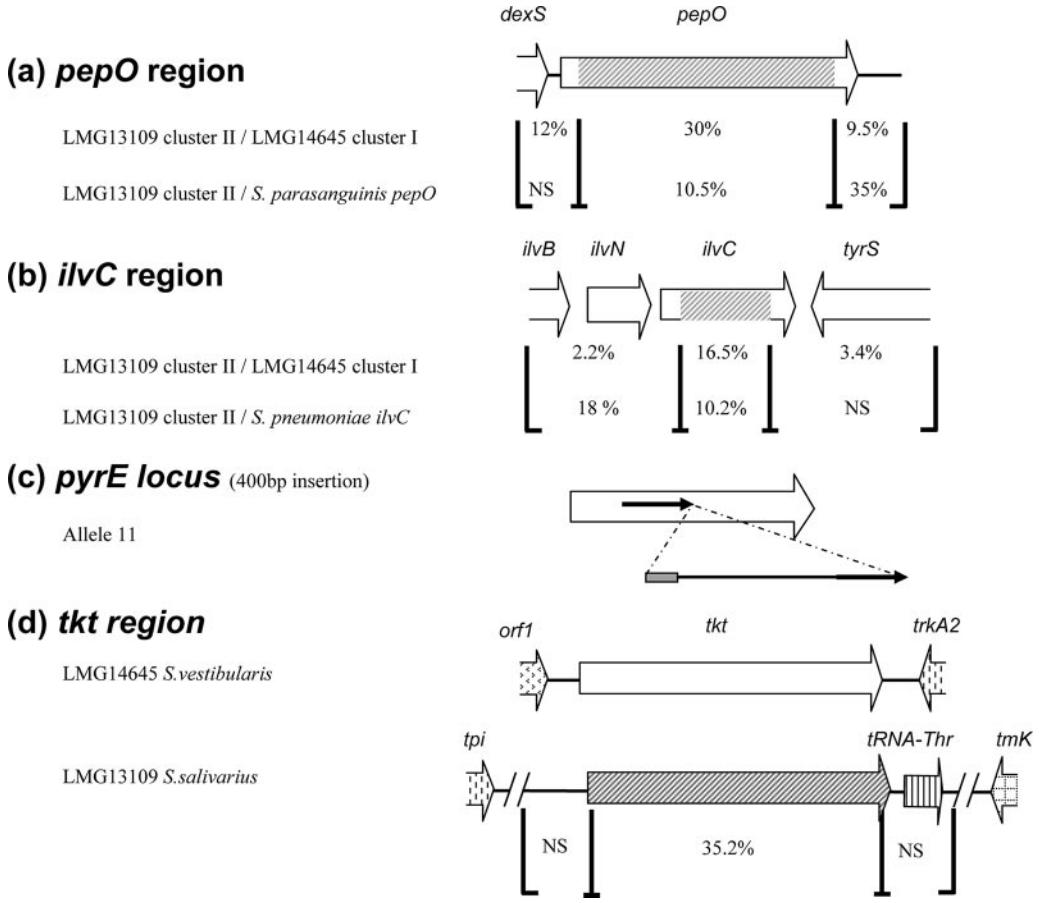

FIG. 5. HGT events detected in *S. salivarius pepO*, *ilvC*, *pyrE*, and *tkt* genes. Regions where there are high and low levels of diversity are indicated by brackets, and the level of diversity is shown for each region. NS, no significant homology. (a) In the *pepO* region, 2,095-bp nucleotide sequences of LMG13109 cluster II and LMG14645 cluster I were compared. The regions were compared with the *S. parasanguinis pepO* region. (b) In the *ilvC* region, 2,765-bp nucleotide sequences of LMG13109 cluster II and LMG14645 cluster I were compared. The regions were compared with the *S. pneumoniae ilvC* region. (c) In allele 11 of the *pyrE* locus, a 234-bp DNA insertion containing 30 bp of *S. salivarius* IS*1139* (gray box) and 204 bp of DNA whose function and origin are unknown is present and is flanked by a 157-bp duplication (arrows). (d) In the *tkt* region, 2,506-bp nucleotide sequences of *S. salivarius* LMG13109 and *S. vestibularis* LMG14645 were compared. *orf1* exhibits the highest level of homology to str0310 from *S. thermophilus*.

quenced. The level of divergence of the *S. salivarius* and *S. vestibularis tkt* gene sequences was 35.2% (Fig. 5d). Phylogenetic analysis showed that two types of *tkt* genes are present in streptococci (Fig. 6). The *tkt* gene of *S. salivarius* belongs to the first group (highest level of identity with *Streptococcus pyogenes* and *Streptococcus agalactiae* genes, 78%), whereas the *tkt* gene of *S. vestibularis* belongs to the second group (highest level of identity with *S. thermophilus* gene, 98%). Further amplification by inverse PCR and sequencing of the flanking regions showed that the genetic organization in the *tkt* region was different in the two species (Fig. 5d). The *S. vestibularis tkt* gene is flanked by an ABC transporter gene (highest level of homology to str0310 from *S. thermophilus*) and *trkA2* (encoding potassium uptake protein); a similar organization occurs in *S. thermophilus*. By contrast, the *S. salivarius tkt* gene is followed by a $tRNA^{Thr}$ gene and is situated in a region flanked by *tpi* (encoding triose phosphate isomerase) and *tmk* (thymidylate kinase), which are adjacent in the *S. thermophilus* genome (data not shown). PCR experiments showed that the genetic organization of the *tkt* region is typical for each species (*S. salivarius* and *S. vestibularis*) (data not shown). The *trkA2* and *tpi* loci are located 170 kb apart in the *S. thermophilus* genome, indicating that a major rearrangement occurred during differentiation of the *S. vestibularis* and *S. salivarius* loci.

DISCUSSION

In the present study, we carried out a comparative sequence analysis of several loci to explore the population structure of *S. salivarius* and *S. vestibularis.* Due to the small number of *S. vestibularis* isolates, an MLST scheme was obtained only for *S. salivarius.* The analysis allowed us (i) to investigate the genetic diversity within *S. salivarius* and *S. vestibularis* isolates and the relationships between them, (ii) to evaluate the relationships of commensal and pathogen strains, and (iii) to characterize extensive HGT in the two populations.

MLST of *S. salivarius*. As a first step in developing an MLST method, the internal fragments of eight loci (*dnaE*, *thrS*, *pyrE*, *ilvC*, *pepO*, *ddlA*, *sodA*, and *glcK*) were amplified from all strains tested. One locus, *tkt*, could not be amplified from *S.*

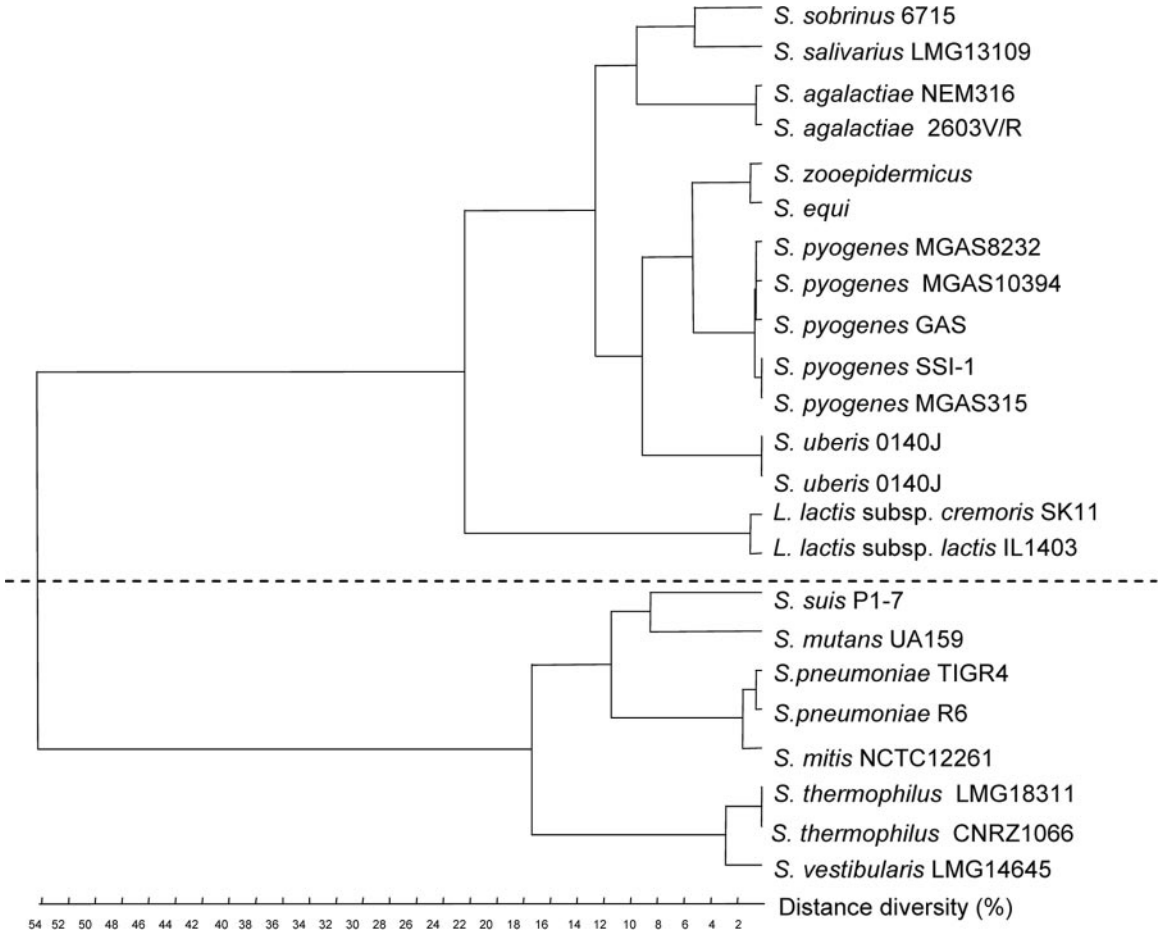

FIG. 6. Additive distance tree for *tkt* genes from streptococci and lactococci. Alignment was performed with ClustalW, and the tree was constructed by the flexible method. The levels of identity between the two clusters range from 54% to 58%, whereas the levels of identity are more than 73% for the genes within each cluster.

salivarius strains. The eight loci were polymorphic, and the maximal levels of divergence of three genes, *ilvC*, *pepO*, and *glcK*, were more than 12%. These high levels of divergence, which could have resulted from interspecific HGT events (see below), prevented use of these three loci in an MLST scheme for *S. salivarius*.

An analysis of alleles and STs showed that the diversity in *S. salivarius* is very high. The variability is comparable to the maximal sequence divergence (5 to 32%) and the mean nucleotide diversity (16.6%) reported for group C and G streptococci and for *S. oralis*, respectively (33, 55). Like *S. salivarius*, these streptococci are considered to be commensals, even though they are sometimes associated with human disease. The level of *S. salivarius* diversity is significantly higher than the levels of diversity of group A streptococci (maximal divergence, 1.4 to 6.1% [17]) and group B streptococci (1.2 to 2.5% variable nucleotide sites [32]). We found that *S. salivarius* isolates from the same individual exhibit a high degree of genetic diversity, as observed previously for several other mucosal streptococci (*S. mitis*, *S. mutans*, and *S. oralis*) (19, 38, 57). The phylogenetic tree of concatenated sequences of *S. salivarius* did not allow us to cluster strains isolated from human blood specifically. We concluded that commensal and potentially pathogenic strains do not belong to distinct *S. salivarius* populations. The lack of clear separation of commensal and clinical *S. salivarius* isolates resembles the findings reported for several other commensal streptococci, such as group C and G streptococci (*S. mitis* and *S. oralis*), which also exhibit opportunistic pathogenicity (33, 55).

Genetic relationship of *S. salivarius* and *S. vestibularis* isolates. *S. salivarius* and *S. vestibularis* are included in the salivarius group of viridans streptococci (18). In 1988, *S. vestibularis* was described as a new oral species (56), while further analysis showed that it is very closely related to *S. salivarius* (34, 46). In this work, we found that the levels of relatedness between *S. vestibularis* and *S. salivarius* are very different at different genetic loci.

Two findings support the notion that *S. salivarius* and *S. vestibularis* should be considered separate species: (i) the strongly supported clusters of *S. vestibularis* alleles at the *glcK*, *pyrE*, *dnaE*, *sodA*, and *ilvC* loci and (ii) the extent of genomic divergence in particular loci, such as the divergence observed for *tkt*. Incidentally, the great differences in the sequence and genetic organization of the *tkt* locus could probably be used to develop a rapid test for discriminating these two bacteria.

By contrast, the lack of resolution of *S. vestibularis* and *S. salivarius* at three loci (*ddlA*, *pepO*, and *thrS*) suggests that there is frequent gene exchange between these two species. Considering the mosaic structure of these three *S. vestibularis* genes and the low levels of divergence found at five other loci (*glcK*, *ilvC*, *pyrE*, *sodA*, and *tkt*), we suggest that *S. vestibularis* may be a recently emerging population which evolves by interspecific gene exchange. However, analysis of a larger sample of *S. vestibularis* strains is necessary to test this hypothesis more stringently and to better define the evolutionary scheme of *S. vestibularis*.

Intraspecific recombination in *S. salivarius* and *S. vestibularis* populations. Analysis of different loci of *S. salivarius* and *S. vestibularis* indicated that the loci have different evolutionary histories. Extensive intraspecific recombinational exchanges should have occurred to explain the features observed.

The noncongruence between allele phylogenetic trees and the low index of association of MLST alleles can be explained by frequent recombination events. The extent of the conflicting phylogenetic results is illustrated by the fact that a strain which has a certain position in one allele tree can occupy a very different position in another allele tree, with both positions strongly supported by bootstrap analysis. The conflicting phylogenetic signals found for seven *S. salivarius* loci in split decomposition analysis, coupled with a low association index value (I^S_A, 0.057), indicate that intraspecific recombination occurs frequently in *S. salivarius* and plays a major role in generating sequence diversity between strains. A specific gene transfer event is revealed by the presence of *pyrE* allele 11 in two strains isolated from human blood 5 years apart (CIP104994 isolated in 1996 and CCHSS4 isolated in 2001). This allele contains a specific DNA insertion consisting of 30 bp of *S. salivarius* insertion sequence IS*1139* (40), followed by 204 bp of DNA whose origin is unknown (Fig. 5c). The events required to generate this allele were unlikely to occur independently twice, suggesting that the allele was exchanged horizontally. In *S. vestibularis*, split decomposition analysis suggested that intragenic recombination occurred at four of the nine loci (not shown). Taken together, these results support the conclusion that intraspecific recombination occurs extensively in the *S. salivarius* population and probably in *S. vestibularis*.

HGT between oral streptococci. The presence of longer branches in *ilvC*, *glcK*, and *pepO S. salivarius* split graphs (see Fig. S2 in the supplemental material) are consistent with the importation of divergent genes from other species (27). This hypothesis is supported by the higher levels of homology of cluster II alleles of *ilvC* and *pepO* loci with *S. pneumoniae* and *S. parasanguinis* alleles, respectively, than with the other *S. salivarius* allele clusters. *S. pneumoniae* and *S. parasanguinis* are oral streptococci which might come in contact with *S. salivarius* and *S. vestibularis* in the buccal cavity. Evaluation of the extent of HGT in *ilvC* and *pepO* genes by sequence analysis indicated that exchange of 725- and 1,783-bp internal gene fragments, respectively, took place (Fig. 5a and b). Differences in chromosomal localization and high levels of nucleotide divergence between *tkt* genes from *S. salivarius* and *S. vestibularis* are also consistent with an HGT event. Detailed cluster analysis of these sequences and the presence of multiple variants in clusters suggest that the transfers are ancient. For example, the transfer of the *ilvC* divergent allele, possibly from the mitis group, likely preceded the emergence of the food species *S. thermophilus* in the salivarius group (4, 28). Notably, alleles of each cluster are still maintained in the population.

The extent of recombination and HGT in *S. salivarius* and *S. vestibularis* populations suggests that these bacteria have efficient mechanisms for gene acquisition and recombination, although they are not known to be naturally competent (6). Natural transformation has been described for *S. pneumoniae* and several viridans streptococci, such as *S. mutans* and *Streptococcus sanguinis* (1, 20, 44), and plays an important role in generating the high levels of genotypic diversity in these species. Recombinational events are responsible for the dissemination of genes encoding virulence factors, such as antibiotic resistance markers, quinolone resistance, immunoglobulin A1 protease, and competence-stimulating peptide (5, 21, 25, 45, 54). Altered penicillin binding protein genes are directly involved in penicillin resistance, and DNA sequences closely related to them appear to have been distributed horizontally between *S. pneumoniae* and viridans streptococci (12, 13, 22). Gene flow from human pathogens to commensal streptococci, described previously for groups A, C, and G (33, 50) and for the oral mitis group (55) and also observed here, may be involved in the evolutionary model of the commensal streptococci, which can be associated with bacterial infections. Recently, phylogenetic analyses of more than 20 streptococcal species revealed several examples of interspecific homologous recombination in housekeeping genes of viridans streptococci (29). Evidence for HGT and recombination events in this study associated *S. salivarius* and *S. vestibularis* with this group of oral streptococci, which have great potential to be receptors of foreign genes.

The absence of clustering of *S. salivarius* strains isolated from blood suggests that these strains are opportunistic pathogens. However, the high potential of *S. salivarius* and *S. vestibularis* for acquisition of new alleles at loci involved in general functioning of the cell and the possible acquisition of antibiotic resistance determinants (23, 51) raise the possibility that strains isolated from human infections acquired DNA that promoted their pathogenic behavior. Further studies are required to examine this possibility.

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