Sequence of the *gtfK* gene of *Streptococcus salivarius* ATCC 25975 and evolution of the *gtf* genes of oral streptococci

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Many strains of oral streptococci secrete glucosyltransferases (GTFs) that polymerize sucrose into glucans that form an integral part of the plaque matrix on the tooth surface. Recently, we reported the cloning of two closely linked GTF-encoding genes (*gtfJ* and *gtfK*) from *Streptococcus salivarius* ATCC 25975 as well as the sequence of *gtfJ*, which encodes a primer-dependent GTF that synthesizes an insoluble product (a GTF-I). In this communication we report the sequence of *gtfK*, which encodes a primer-dependent GTF that synthesizes a soluble product (a GTF-S), as well as the sequence of a small downstream open reading frame of unknown function. The deduced sequence of GtfK was compared with those of seven other streptococcal Gtfs and an unrooted phylogenetic tree constructed. This analysis suggested that Gtfs with similar product specificities do not form phylogenetic clusters and was consistent with currently accepted phylogenetic schemes. The tree was tested by constructing a series of 'sub-trees' from different blocks of the alignment. Evidence was obtained for recombination events involving *gtfB* and *gtfC* from *S. mutans* GS-5, *gtfJ* and *gtfK* from *S. salivarius*, as well as the *gtfI* genes from *S. downei* and *S. sobrinus*. The recombination events between *gtfB* and *gtfC*, and between the two *gtfI* genes, were confirmed by examining divergences at silent sites.

Introduction

Members of the mutans and sanguis groups of streptococci, as well as *Streptococcus salivarius* strains, synthesize extracellular glucosyltransferases (GTFs) that are considered to be important virulence factors in the aetiology of dental caries. The GTFs are highly hydrophilic monomeric proteins of approximate M_r 160 000 that form glucans by the polymerization of the glucose moiety of sucrose and the release of fructose. It is these glucans that promote dental caries by conferring adherent and anti-diffusion properties on dental plaque (Gibbons, 1968). In most cases the GTFs require a primer glucan for activity. These primer-dependent GTFs either extend the length of, or add branching points to, a pre-existing glucan. It is the nature of the linkages in these glucans that determines their solubility, for those polymers that are predominantly α -(1→3)-

linked are insoluble whilst those that are predominantly α -(1→6)-linked are soluble (Walker, 1978; Rölla *et al.*, 1983; Walker & Jacques, 1987). The GTFs that produce these glucans are appropriately designated GTF-Is and GTF-Ss respectively.

To date, the sequences of seven streptococcal GTF-encoding genes have been published (Table 1). It is clear that all these genes are closely related and that their deduced amino acid sequences share a common domain structure in which the N-terminal two-thirds of the

Table 1. Oral streptococcal GTFs of known sequence

Enzyme	Strain	Class	Reference
GtfB	<i>S. mutans</i> GS-5	GTF-I*	Shiroza <i>et al.</i> (1987)
GtfC	<i>S. mutans</i> GS-5	GTF-SI*	Ueda <i>et al.</i> (1988)
GtfD	<i>S. mutans</i> GS-5	GTF-S	Honda <i>et al.</i> (1990)
GtfI (dn)	<i>S. downei</i> MFe28 (formerly <i>S. sobrinus</i>)	GTF-I	Ferretti <i>et al.</i> (1987)
GtfI (sb)	<i>S. sobrinus</i> 6715	GTF-I	Abo <i>et al.</i> (1991)
GtfS	<i>S. downei</i> MFe28	GTF-S	Gilmore <i>et al.</i> (1990)
GtfJ	<i>S. salivarius</i> ATCC 25975	GTF-I	Giffard <i>et al.</i> (1991)

* These enzymes are reported to synthesize mixtures of soluble and insoluble polymers.

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Abbreviation: GTF, glucosyltransferase.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number Z11872 (*gtfK* and *orfI*).

enzyme, designated the 'catalytic domain', is responsible for the cleavage of sucrose (Ferretti *et al.*, 1987). The C-terminal one-third, on the other hand, will bind to glucan and is known as the 'glucan-binding domain' (Ferretti *et al.*, 1987; Mooser & Wong, 1988; Kobayashi *et al.*, 1989). An active-site aspartic acid residue has been located in the catalytic domain (Mooser & Iwaoka, 1989; Mooser *et al.*, 1991), while the glucan-binding domain is composed of a series of glycine-rich repeat sequences that exhibit similarity to the repeats in the glucan-binding protein from *Streptococcus mutans* (Banas *et al.*, 1990) as well as the ligand-binding domains in *Clostridium difficile* toxin A and the lysins from *Streptococcus pneumoniae* (Wren, 1991).

In this communication, we report the sequencing of *gtfK*, which encodes a primer-dependent GTF-S in *S. salivarius* ATCC 25975 (Pitty *et al.*, 1989), and the comparison of the deduced amino acid sequence of GtfK with seven other Gtfs. A plausible multiple alignment of the central portion of the Gtf sequences was constructed and used to deduce a phylogenetic tree.

Methods

Strains and growth conditions. Plasmids were maintained in *Escherichia coli* strain NM522 (Gough & Murray, 1983) and derivatives of bacteriophage λ were grown on *E. coli* strain LE392 (Murray *et al.*, 1977). All *S. salivarius* DNA was derived from λ -A33, which has been described previously (Pitty *et al.*, 1989; Giffard *et al.*, 1991). *E. coli* cells were routinely grown on Luria Bertani (LB) medium (Miller, 1972) supplemented with ampicillin ($100 \mu\text{g ml}^{-1}$), isopropyl β -D-thiogalactoside (IPTG) (1 mM), or 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) ($100 \mu\text{g ml}^{-1}$) as appropriate.

DNA sequence determination. All sequence determinations were carried out using the chain-termination method (Sanger *et al.*, 1977). DNA that was to be sequenced was first subcloned into either or both of the ϕ 101-containing phagemids pIB130 or pIB131 (IBI corporation), using routine procedures (Maniatis *et al.*, 1982). The subcloned fragments are indicated in Fig. 1. In general, sequencing in the first direction was accomplished using a series of nested deletions constructed by the exonuclease III method of Henikoff (1984), and then confirmed in the other direction using custom-synthesized oligonucleotide primers. However, on occasion, sequencing was carried out in both directions using custom-synthesized primers. Templates were either single stranded or double stranded. Single-stranded templates were prepared using the helper phage M13K07 by the method recommended by Pharmacia for their pTZ series of phagemids, while double-stranded templates were prepared by the method of Birnboim & Doly (1979) and further purified by ultra-centrifugation through CsCl gradients (Maniatis *et al.*, 1982). All sequencing reactions were carried out using the Pharmacia T7 sequencing kit according to the manufacturer's instructions. Complete sequences were obtained in both directions.

Sequences were assembled using the IBI Pustell sequence analysis software version 2.03. Database searches were carried out using the 'mfa' program (Pearson & Lipman, 1988) in the Australian National Genome Information Service (ANGIS) based at the University of Sydney, Australia.

Multiple alignments and phylogenetic tree constructions. Both were carried out using the 'ClustalV' program (Higgins *et al.*, 1992) in the

ANGIS facility at the University of Sydney. The alignment parameters used are described in the appropriate sections. Tree construction was done using the neighbour-joining method (Saitou & Nei, 1987). In all cases, positions where there was a gap in any of the sequences were ignored, and branches were lengthened to account for multiple substitutions according to the algorithm of Kimura (1983). Confidence limits were calculated by means of the 'bootstrapping' method of Felsenstein (1985) using 1000 samples.

Divergence analyses. The divergences at silent sites (changes that do not alter the identity of the encoded amino acid) and replacement sites (changes that do alter the identity of the encoded amino acid) for pairs of coding sequences were calculated using the program 'Diverge' available within the Genetics Computer Group (GCG) collection of sequence analysis programs accessed through the ANGIS facility. This program makes use of the algorithm and the 'categories' of nucleotide changes described by Perler *et al.* (1980).

The input for the analyses were in-frame nucleic acid sequence alignments. The nucleic acid alignments were either derived from the amino acid sequence alignments, or were generated by aligning the nucleic acid sequences directly using the 'Best-Fit' program in the GCG package. The presence of gaps in the alignment, and also the necessity to discern local levels of divergence, required that the analyses be carried out using discrete blocks of alignment. The precise conditions used in each experiment are described in the relevant sections.

Results

The sequence of *gtfK* and the downstream region

We have previously suggested that *gtfK*, which encodes a primer-dependent GTF-S activity, lies 210 bp downstream from *gtfJ*, which encodes a primer-dependent GTF-I activity, on the *S. salivarius* chromosome (Giffard *et al.*, 1991; Fig. 1). As we had only reported the 5' end of the putative *gtfK* gene, DNA sequence determination was carried out from the limit of this previously published sequence to the right-hand end of the cloned DNA (Fig. 1). It was found that the beginning of the putative *gtfK* could be extended to give an open reading frame (ORF) of 4797 bp which encoded a polypeptide of M_r 176466 that could be readily aligned with other GTF sequences (see below). The *gtfK* was also preceded by putative ribosome-binding site and promoter elements (Fig. 2).

Fig. 1. Physical map of the *gtfJK* locus, modified from Giffard *et al.* (1991). Filled areas represent the coding regions and the arrows the direction of transcription. The subclones used for sequencing *gtfK* and *orf1* are also shown. H, *Hind*III; E, *Eco*RI; B, *Bgl*II; S, *Sac*I.

The C-terminal third of all sequenced GTFs is composed of a complex series of repeated sequences that have been shown to be associated with glucan-binding. These repeats have been divided into four classes (A–D) (Ferretti *et al.*, 1987; Gilmore *et al.*, 1990; Giffard *et al.*, 1991) although the different classes do exhibit a certain level of similarity. Of these classes, the ‘A’ repeat has been found in multiple copies in all GTFs sequenced to date. The GtfK sequence was no exception as it contained seven ‘A’ repeats, of which five were immediately followed by sequences that showed similarity to the ‘D’ repeats of GtfJ and thus have been classified accordingly. The sequences of the repeats in GtfK and the location of the repeat classes in all known GTF sequences are shown in Fig. 3.

The GTFs are secreted enzymes and so would be expected to possess cleavable N-terminal signal sequences. Ferretti *et al.* (1987) have carried out N-terminal sequence analysis on the mature secreted form of Gtf-I from *S. downei* and have shown that a signal sequence cleavage site exists downstream of a hydrophobic region that follows von Heijne’s ‘–1, –3 rule’ (von Heijne, 1983). The GtfK sequence has an homologous hydrophobic stretch of amino acids followed by two alanine residues that occur at the ‘–1, –3’ sites in GtfI and so is also likely to be cleaved at this position (Fig. 4).

An ORF of unknown function follows gtfK

The sequenced DNA also defined another ORF 141 bp downstream from *gtfK* that we have designated *orfI* (Fig. 1). This ORF is 345 bp in length and is preceded by putative ribosome-binding site and promoter elements (Fig. 2). It defines a highly hydrophobic polypeptide of M_r 12884. The deduced amino-acid sequence was used to search available sequence databases. While no examples of clear similarity were found, the sequence did show low levels of similarity with a number of membrane-associated proteins (data not shown), although by the criteria of von Heijne (1983) there was no clear evidence of any cleavable translocation signal sequence. The function of the product of *orfI* remains unknown.

Multiple alignment and phylogenetic comparison of available Gtf sequences

In order to investigate the relationship between different Gtf sequences, it was first necessary to construct a plausible multiple alignment of a portion of the eight Gtf sequences. This was necessary as two regions within the eight sequences aligned extremely poorly (data not shown). Not unexpectedly, the first of these was the C-terminal glucan-binding domain with its apparent large

number of duplications and rearrangements (Fig. 3). The second was a region of approximately 200 amino acids immediately following the signal sequence which was found to be highly variable and difficult to align reliably. Omission of both these domains from the ClustalV analysis yielded a multiple alignment of the eight Gtf sequences that contained a relatively small number of gaps. In order to obtain the ‘best’ alignment possible, the gap penalties were progressively modified. Significant adjustment of the pairwise gap penalty had no effect on the results, so attention was given to adjusting the multiple alignment fixed gap penalty and floating gap penalty, which control the opening of a gap and lengthening of a gap respectively. Progressively altering these gap penalties had only a slight effect on the multiple alignment until certain values were reached, at which point essential gaps were not opened and the multiple alignment became implausible.

Different alignments were tested by building phylogenetic trees using the neighbour-joining method (Saitou & Nei, 1987) and comparing the bootstrapping confidence limits, topologies and branch lengths. In all plausible alignments, the confidence limits were 100% for all branch orders except for the shortest, which varied between 75% and 90%, the topologies were all identical, and branch lengths the same within 5%. By subjective judgment, the alignment obtained by using a fixed gap penalty of 10 and a floating gap penalty of 30 was chosen for further analysis (Fig. 5).

A phylogenetic tree was constructed from this alignment excluding all positions where gaps occurred (Fig. 6). The topology was tested by bootstrapping (Felsenstein, 1985) using 1000 samples. All the branching orders gave a confidence limit of 100% except for the separation of GtfJ, GtfK and GtfD from the other sequences which gave a confidence limit of 79.3%.

Two significant observations could be drawn from the phylogenetic analysis shown in Fig. 6. First, if one excluded the groupings of GtfB/GtfC, and the two GtfIs (both of which are identical over much of their lengths – see below), there was no clustering of enzymes that make similar glucan products. Neither GtfJ and the two GtfIs, which make insoluble products, nor GtfS, GtfD and GtfK, which make soluble products, form natural groups. Since insoluble glucans are primarily α -(1→3)-linked while soluble glucans are α -(1→6)-linked (Walker & Jacques, 1987; Pitty *et al.*, 1989; Gilmore *et al.*, 1990), it can be inferred that different product linkage specificities may have evolved more than once. Secondly, there was little difference between the maximum divergence between sequences from within the mutans group and the divergence between the sequences from *S. salivarius* and the mutans group. This indicated that in the absence of inter-species gene transfer, the existence of multiple

10										20										30										40																													
GATAAAARATA TACTTAAANT AAGTTTTAT AACTTTTTT																																																											
50										60										70										80										90										100									
GATAATAAAA AATGATATAA TAAGTAAGTC TTTAGATGTT AGATTTTAAAG ACAAARATA																																																											
-35										-10																																																	
110										120										130										140										150																			
TATTGGGGAA										AAATGAAT										ATG GAA AAT AAA GTA CGC TAT AAA CTG CAC AAA GTG																																							
RBS										Met Glu Asn Lys Val Arg Tyr Lys Leu His Lys Val																																																	
										gtfK→																																																	
160										170										180										190										200																			
AAG AAG CAG TGG GTT ACT TTG GCT GCT GCT TCA GTA GCT TTG GCT ACC ATT																																																											
Lys Lys Glu Trp Val Thr Leu Ala Val Ala Ser Val Ala Leu Ala Thr Ile																																																											
210										220										230										240										250																			
GTT GGT GGT AGT GTT GCC ACA TCT TCA CTC GCT TCG GCA GAA GAA ACA AAT																																																											
Val Gly Gly Ser Val Ala Thr Ser Ser Ser Leu Ala Ser Ala Glu Glu Thr Asn																																																											
260										270										280										290										300																			
AAC TCT AAT GGA TCA CCT TCA ACA ACT ACG GTC GGC GAA AAT ACA AAT CCT																																																											
Asn Ser Asn Gly Ser Pro Ser Thr Thr Thr Thr Val Gly Glu Asn Thr Asn Pro																																																											
310										320										330										340										350																			
GTG GTT GAA AAA GAG GTA GGC ACA ACA ACT GAT GTT GCA AAT ACT TCA AAT																																																											
Val Val Glu Glu Lys Glu Val Gly Thr Thr Thr Thr Glu Val Ala Asn Thr Ser Asn																																																											
360										370										380										390										400										410									
GCC ACA ACA ACA GAG CGA GCA GAA GTT ACT GCT GAC AAA CCA GCT GGA ACG																																																											
Ala Thr Thr Thr Glu Arg Ala Glu Val Thr Ala Arg Lys Pro Ala Gly Thr																																																											
420										430										440										450										460																			
ACA GTT CAA CCA AAT TCA GGA ACA ACT AGT AGT AGT AGA GCT GCA GCA GTA GAG																																																											
Thr Val Glu Lys Pro Asn Ser Gly Thr Thr Thr Ser Asp Arg Ala Ala Ala Val Glu																																																											
470										480										490										500										510																			
GTT GAA GCC AAA CCT GAA ACG ACT GCT AAG CCA GAA GTT GCT ACT AAA CCA																																																											
Val Glu Ala Lys Pro Glu Thr Thr Thr Ala Lys Pro Glu Val Ala Thr Lys Pro																																																											
520										530										540										550										560																			
GAA ACA GCT ACA ACA TCA GAA GTC GCT GCA AAT GCT GGA GTA GCT GCT CCA																																																											
Glu Thr Thr Ala Thr Thr Ser Glu Val Ala Ala Asn Ala Gly Val Ala Ala Pro																																																											
570										580										590										600										610																			
ACG ACT GAG AAG TCT AAA AAG CTT TCT GAA GCC GAA ATT AAG GCT GCA GTC																																																											
Thr Thr Glu Lys Ser Lys Glu Leu Ser Glu Ala Glu Ile Lys Ala Ala Val																																																											
620										630										640										650										660																			
TCA CTC GAT AAT ATT AAA AAA GAA AAT GGT AAA TAT TAT TAT CTT TTA																																																											
Ser Leu Asp Asn Ile Lys Lys Glu Lys Asp Gly Lys Tyr Tyr Tyr Leu Leu																																																											
670										680										690										700										710																			
GAA GAT GGA TCA CAC AAG AAA AAC TTT GCC ATT ACT GTA AAT GGT CAG GTC																																																											
Glu Asp Gly Ser His Lys Lys Asn Phe Ala Ile Thr Val Asn Gly Glu Val																																																											
720										730										740										750										760																			
CTT TAT TTT GAT GAA AAT GGT GCC CTT TCA AGC ACA TCA ACG TAT TCA TTT																																																											
Leu Tyr Phe Asp Glu Asn Gly Ala Leu Ser Ser Thr Ser Thr Thr Ser Phe																																																											
770										780										790										800										810																			
ACA CAA GAA ACA ACA AAT CTA GTC ACT GAT TTT ACA AAA AAT AAT GCT GCC																																																											
Thr Glu Glu Thr Thr Asn Leu Val Thr Asp Phe Thr Lys Asn Asn Ala Ala																																																											
820										830										840										850										860																			
TAC GAT TCA ACT AAA GCA AGC TTT GAA CTT GTT GAT GGT TAT TTG ACA GCA																																																											
Tyr Asp Ser Thr Lys Ala Ser Phe Glu Leu Val Asp Gly Tyr Leu Thr Ala																																																											
870										880										890										900										910										920									
GAC AGT TGG TAT CGT CCA AAA GAA ATC CTT GAA GCT GGT ACA ACT TGG AAA																																																											
Asp Ser Thr Tyr Arg Pro Lys Glu Ile Leu Glu Ala Gly Tyr Tyr Thr Lys																																																											
930										940										950										960										970																			
GCG TCA ACT GAA AAA GAT TTT CGT CCA CTT TTG ATG TCT TGG TGG CCT GAT																																																											
Ala Ser Thr Glu Lys Asp Thr Arg Pro Leu Leu Met Ser Trp Trp Trp Pro Asp																																																											
980										990										1000										1010										1020																			
AAG GAT ACT CAA GTA GCT TAC TTG AAT TAC ATG ACT A																																																											

1490				1500				1510				1520				1530			
TTT	GAT	GGT	ATA	CGT	GTT	GAT	GCT	GTG	GAT	AAT	GTT	AGC	GTT	GAC	ATG	CTT			
Phe	Asp	Gly	Ile	Arg	Val	Asp	Ala	Val	Asp	Asn	Val	Ser	Val	Asp	Met	Leu			
1540				1550				1560				1570				1580			
CAA	TTA	GTG	TCT	TCA	TAC	ATG	AAG	GCT	GCA	TAC	AAG	GTT	ART	GAG	TCG	GAA			
Gln	Leu	Val	Ser	Ser	Tyr	Met	Lys	Ala	Val	Asp	Val	Asn	Glu	Ser	Glu	Ala			
1590				1600				1610				1620				1630			
GCT	CGT	GCC	CTT	CGC	AAT	ATC	TCT	ATT	CTT	GAA	GCA	TGG	GCA	CAC	AAT	GAC			
Ala	Arg	Ala	Leu	Ala	Asn	Ile	Ser	Ile	Leu	Glu	Ala	Trp	Ser	His	Asn	Asp			
1640				1650				1660				1670				1680			
CCA	TAT	TAT	GTG	AAT	GAG	CAC	ACC	ACA	GCA	GCT	CTT	TCT	ATG	GAT	AAT	GGT			
Pro	Tyr	Tyr	Val	Asn	Glu	His	Asn	Thr	Ala	Ala	Leu	Ser	Met	Asp	Asn	Gly			
1690				1700				1710				1720				1730			
CTC	CGT	TTG	TCA	ATT	GTT	CAC	GGA	TTA	ACT	CGT	GCA	GTA	ACT	AAT	AAA	GGT			
Leu	Arg	Leu	Ser	Ile	Val	His	Gly	Leu	Thr	Arg	Pro	Val	Thr	Asn	Lys	Gly			
1740				1750				1760				1770				1780			
ACA	GGT	GCA	CGT	AAT	GCA	TCA	ATG	AAG	GAC	CTT	ATT	AAC	GGA	GGT	TAT	TTC			
Thr	Gly	Ala	Arg	Asn	Ala	Ser	Met	Lys	Asp	Leu	Ile	Asn	Gly	Gly	Tyr	Phe			
1790				1800				1810				1820				1830			
GGT	CTC	TCA	AAC	CGT	GCA	GAA	GTC	ACT	TCA	TAT	GAT	CAA	CTT	GGT	TTT	GCA			
Gly	Leu	Ser	Asn	Arg	Ala	Glu	Val	Thr	Ser	Tyr	Asp	Gln	Leu	Ser	Phe	Ala			
1840				1850				1860				1870				1880			
ACC	TAC	CTC	TTT	GTT	CGT	GCA	CAT	GAC	TCA	GAA	GTT	CAA	ACG	GTT	ATC	GCA			
Thr	Tyr	Leu	Phe	Val	Arg	Ala	His	Asp	Ser	Glu	Val	Gln	Thr	Val	Ile	Ala			
1890				1900				1910				1920				1930			
GAC	ATT	ACT	AGT	AAA	AAA	ATT	GAT	CCT	ACA	ACA	GAT	GGT	TTC	ACT	TTC	ACA			
Asp	Ile	Ile	Ser	Ser	Lys	Lys	Ile	Asp	Pro	Thr	Thr	Asp	Gly	Phe	Thr	Phe			
1940				1950				1960				1970				1980			
TTA	GAT	CAA	TTG	AAA	CAA	GCT	TTC	GAT	ATT	TAT	AAT	GCT	GAT	ATG	TTG	AAA			
Leu	Asp	Gln	Leu	Lys	Gln	Ala	Phe	Asp	Ile	Tyr	Asn	Ala	Asp	Met	Leu	Lys			
1990				2000				2010				2020				2030			
GTT	GAT	AAG	GAA	TAT	AGC	CAT	TCA	AAT	ATT	CCT	GCT	GCC	TAT	GCT	TTG	ATG			
Val	Asp	Lys	Glu	Tyr	Thr	His	Ser	Asn	Ile	Pro	Ala	Ala	Tyr	Ala	Leu	Met			
2040				2050				2060				2070				2080			
CIT	CAA	ACA	ATG	GGT	GGC	GCA	ACA	CGT	GTT	TAT	TAT	GGA	GAT	TTG	TAT	ACT			
Leu	Gln	Thr	Met	Gly	Ala	Ala	Thr	Arg	Val	Tyr	Tyr	Gly	Asp	Leu	Tyr	Thr			
2090				2100				2110				2120				2130			
GAT	AAT	GGT	CAA	TAC	ATG	GCT	AAG	AAA	TCA	CCA	TAC	TTT	GAT	CAA	ATC	ACA			
Asp	Asn	Gly	Gln	Tyr	Met	Ala	Lys	Lys	Ser	Pro	Tyr	Phe	Asp	Gln	Ile	Thr			
2140				2150				2160				2170				2180			
ACT	CTT	CTT	AAA	GGC	GGT	CCT	AAG	TAT	GTA	GCT	GGT	GGT	CAA	ACA	TCT	TAT			
Thr	Leu	Leu	Lys	Ala	Arg	Pro	Lys	Tyr	Val	Ala	Gly	Val	Ala	Ala	Ala	Ala			
2190				2200				2210				2220				2230			
ATC	CAC	A																	

2910	2920	2930	2940	2950	2960
AGC AAA AAC AAT TAC GGT TCA AAA GAA GAC CTT CTT GAT GCC CTC AAG	Ser Lys Asn Asn Lys Tyr Gly Ser Lys Glu Asp Leu Arg Asp Ala Leu Lys				
3000	3010	3020	3030	3040	3050
GCT CTT CAC AAA CAA GGT ATT ATT CAG GTC ATT GCT GAC TGG GTT CCA GAC CAA	Ala Leu His Lys Lys Gln Gly Ile Gln Val Ile Ala Asp Trp Val Pro Asp Thr				
3060	3070	3080	3090	3100	3110
TTG TAC ACC CTT CCA GGT AAA GAA GTA GTG ACT GCT ACG CGT ACT GAT ACA	Leu Tyr Thr Leu Pro Gly Lys Glu Val Val Thr Ala Thr Arg Thr Asp Thr				
3120	3130	3140	3150	3160	
CAT GGT AAG GTC CTT GAT ACT AGC TTG GTT AAT AAA CTT TAT GTG ACA	His Gly Lys Val Leu Asp Asp Thr Ser Leu Val Asn Lys Leu Tyr Val Thr				
3170	3180	3190	3200	3210	
AAT ACC AAG TCA TCA GGT AAT GAT TTC CAA GCC CAG TAC GGT GGT GCC TTC	Asn Thr Lys Ser Ser Gly Asn Asp Phe Gln Ala Gln Tyr Gly Gly Ala Phe				
3220	3230	3240	3250	3260	
CTC GAC AAA CTT CAA AAA CTT TAT CAA GAA ATC TTC AAA GAA GTT ATG GAA	Leu Asp Lys Leu Gln Lys Leu Tyr Pro Glu Ile Phe Lys Glu Val Met Glu				
3270	3280	3290	3300	3310	
GCG TCT GGT AAG ACA ATT GAT CCA TCT GTG AAG ATT AAA CAG TGG GAA GCT	Ala Ser Gly Lys Thr Ile Asp Pro Ser Val Lys Ile Lys Gln Trp Glu Ala				
3320	3330	3340	3350	3360	
AAA TAC TTT AAC GGT ACA AAC ATC CAA AAA CGT GGT TCT GAC TAT GTT CTC	Lys Tyr Phe Asn Gly Thr Asn Ile Gln Lys Arg Gly Ser Asp Tyr Val Leu				
3370	3380	3390	3400	3410	
AGC GAT GGT AAA TTG TAC TTC ACC GTT AAC GAT AAA GGT ACC TTC CTC CCA	Ser Asp Gly Lys Lys Leu Tyr Thr Val Asn Asp Lys Gly Thr Phe Leu Pro				
3420	3430	3440	3450	3460	3470
GCA GCC TTG ACT GGT GAT ACA AAA GCT AAG ACT GGT TTT GCC TAT GAT GGT	Ala Ala Leu Thr Gly Asp Thr Lys Ala Lys Thr Gly Phe Ala Tyr Asp Gly				
3480	3490	3500	3510	3520	
ACA GGT GTA ACT TAT TAC ACA ACA TCA GGT ACT CAA GCT AAG AGT CAG TTT	Thr Gly Val Thr Tyr Thr Thr Ser Gly Thr Gln Ala Lys Ser Gln Phe				
3530	3540	3550	3560	3570	
GTA ACT TAT AAC GGT AAG CAA TAC TAC TTT AAC GAT AAG GGT TAC CTT GTA	Val Thr Tyr Asn Gly Lys Gln Tyr Phe Asn Asp Lys Gly Tyr Val Leu Val				
3580	3590	3600	3610	3620	
ACT GGT GAA CAA ACT ATC GAT GGT TCT AAC TAT TTC TTC CCA AAT GGT	Thr Gly Glu Gln Thr Ile Asp Gly Ser Asn Tyr Phe Phe Leu Pro Asn Gly				
3630	3640	3650	3660	3670	
GTC ATG TTT ACT GAT GGT AGA AAA AAT GCC AAA GGT CAA TCA TTG GTT	Val Met Phe Thr Asp Gly Val Arg Lys Asn Ala Lys Gly Gln Ser Leu Val				
3680	3690	3700	3710	3720	
TAT GGT AAG TCA GGT AAA TTG ACG ACT CAA ACT GGT TGG AAA GAA GTT ACC	Tyr Gly Lys Ser Gly Lys Leu Thr Thr Gln Thr Thr Lys Glu Val Thr				
3730	3740	3750	3760	3770	
GTT AAA GAT GAT AGT GGT AAG GAA GAG AAA TTC TAC CAA TAC TTC TAC AAG	Val Lys Asp Asp Ser Gly Lys Glu Glu Lys Phe Tyr Gln Tyr Phe Phe Lys				
3780	3790	3800	3810	3820	
GGT GGT ATC ATG GCT ACT GGT TTG ACT GAA GTA GAA GGT AAG GAA AAA TAC	Gly Gly Ile Met Ala Thr Gly Leu Thr Glu Val Glu Gly Lys Glu Lys Tyr				
3830	3840	3850	3860	3870	
TTC TAT GAT AAT GCT TAC CAA GCT AAA GGT GTC TTC GTT CCT ACA AAA GAC	Phe Tyr Asp Asn Gly Tyr Gln Ala Lys Gly Val Phe Val Pro Thr Lys Asp				
3880	3890	3900	3910	3920	
GGC CAT TTA ATC TTC TTC TGT GGT GAT TCA GGC GAA CGT AAG TAC TCA GGC	Gly His Leu Met Phe Phe Cys Gly Asp Ser Gly Glu Arg Lys Tyr Ser Gly				
3930	3940	3950	3960	3970	3980
TTT TTT GAA CAA GAT GGT AAT TGG TAC TAC GCC AAT GAT AAA GGC TAT GTT	Phe Phe Glu Gln Asp Gly Asn Trp Tyr Tyr Ala Asn Asp Lys Gly Tyr Val				
4000	4010	4020	4030	4040	4050
GCT ACT GGG TTT ACT AAG GTA GGT AAA CAA AAC CTT TAC TTC AAT GAA AAA	Ala Thr Gly Phe Thr Lys Val Gly Lys Gln Asn Leu Tyr Phe Asn Glu Lys				
4060	4070	4080	4090	4100	4110
GGT GTT CAA GTT AAG AAT GCT TTC TTC CAA GTT GGT GAT GCT ACA TAC TAT	Gly Val Gln Val Lys Asn Arg Phe Phe Gln Val Gly Asp Ala Thr Tyr Tyr				
4120	4130	4140	4150	4160	4170
GCA AAT AAC GAA GGT GAC GTT CTT CTT GGT GCG CAA ACC ATC AAT GCA GAC	Ala Asn Asn Glu Gly Asp Val Leu Arg Gly Ala Gln Thr Ile Asn Gly Asp				
4180	4190	4200	4210	4220	4230
GAA CTC TAC TTT GAT GAA TCA GGT AAA CAA GTT AAA GGT GAG TTC GTG AAC	Glu Leu Tyr Phe Asp Glu Ser Gly Lys Gln Val Lys Gly Glu Phe Val Asn				
4240	4250	4260	4270	4280	
AAT CCT GAT GGT ACG ACT TCA TAC TAC GAT GCA ATC ACA GGT GTG AAA CTT	Asn Pro Asp Gly Thr Thr Ser Tyr Tyr Asp Ala Ile Thr Gly Val Lys Leu				
4290	4300	4310	4320	4330	
GTT GAT ACA TCA CTA GTA GTT GAT GGT CAA ACC TTT AAT GTC GAT GCT AAG	Val Asp Thr Ser Leu Val Val Asp Gly Gln Thr Phe Asn Val Asp Ala Lys				
4340	4350	4360	4370	4380	
GGT GTT GTG ACT AAG GCA CAT ACA CCA GGC TTC TAT ACT ACT GGG GAC AAC	Gly Val Val Thr Lys Ala His Thr Pro Gly Phe Tyr Thr Thr Thr Gly Asp Asn				
4390	4400	4410	4420	4430	
ATC AAT GGT CAA CAC CTC TAC TTT GAT GCT AAT GGT CGC CAA GTT AAG GGT	Ile Asn Gly Gln His Leu Tyr Phe Asp Ala Asn Gly Arg Gln Val Lys Gly				
4440	4450	4460	4470	4480	4490
GGC TTC GTA ACG AAC ACT GAC GGT AGT CPT TCA TTC TAC CAC TGG AAT ACC	Gly Phe Val Thr Asn Thr Asp Gly Ser Arg Ser Phe Tyr His Trp Asn Thr				
4500	4510	4520	4530	4540	
GGT GAT AAA TTG GTA TCA ACC TTC TTC GCT ACC GGT CAT GAT AGA TGG TAT	Gly Asp Lys Leu Val Ser Thr Phe Phe Val Ser Thr Gly His Asp Arg Trp Tyr				
4550	4560	4570	4580	4590	
TAT GCT GAT GAT AGG GGT AAT GTC GTT ACA GGA GCT CAA GTT ATC AAC GGT	Tyr Ala Asp Asp Arg Gly Asn Val Val Thr Gly Ala Gln Val Ile Asn Gly				
4600	4610	4620	4630	4640	
CAA AAA CTC TTC TTT GAT ACT GAT GGT AAG CAG GGT AAA GGT GCT TTC GCA	Gln Lys Leu Phe Phe Asp Thr Asp Gly Lys Gln Val Lys Gly Ala Phe Ala				
4650	4660	4670	4680	4690	
ACA AAT GCG AAT GGT AGT CGC TCA TAC TAT TGG AAT ACT GGT AAT AAG	Thr Asn Ala Asn Gly Ser Arg Ser Tyr Tyr His Trp Asn Thr Gly Asn Lys				
4700	4710	4720	4730	4740	
TTA GTA TCA ACC TTC TTC ACT AGT GGA GAT AAC AAC TGG TAT TAT GCA GAT	Leu Val Ser Thr Phe Phe Thr Ser Gly Asp Asn Asn Trp Tyr Tyr Ala Asp				
4750	4760	4770	4780	4790	
GCC AAG GGT GAA GTT GTT GTT GGC GAA CAG ACG ATC AAC GGT CAA CAC CTT	Ala Lys Gly Glu Val Val Val Gly Glu Gln Thr Ile Asn Gly Gln His Leu				
4800	4810	4820	4830	4840	
TAC TTT GAC CAA ACT GGT AAG CAA GTG AAG GGG GCA ACT GCT ACG AAT CCT	Tyr Phe Asp Gln Thr Gly Lys Gln Val Lys Gly Ala Thr Ala Thr Asn Pro				
4850	4860	4870	4880	4890	
GAT GGC TCA ATC TCT TAC TAT GAT GTT CAT ACA GGG GAA AAG GCT ATT AAT	Asp Gly Ser Ile Ser Tyr Tyr Asp Val His Thr Gly Glu Lys Ala Ile Asn				
4900	4910	4920	4930	4940	4950
CGT TGG GTT AAA ATT CCT TCA GGG CAA TGG GTA TAC TTC AAC GCT CAA GGA	Arg Trp Val Lys Ile Pro Ser Gly Gln Trp Val Tyr Phe Asn Ala Lys Thr Gln				
4960	4970	4980	4990	5000	5010
AAA GGC TAC GTG TCA AAT TAA G AGTAATGAAT GAAAGAATC AATCATTCTT GTGGT	Lys Gly Tyr Val Ser Asn End				
5020	5030	5040	5050	5060	5070
TGCTT TTTTCGTAAA AAATGTCTA ACTTTTATAC ATTCAAGTCA TTTCTCTGGG TAAAA	-10				
5080	5090	5100	5110	5120	
AAT ACT ATT GAA AGT CGG ATA GAT TAC TCG ATT CTC CTG CCA GTT CTC ATA	Asn Thr Ile Glu Ser Arg Ile Asp Tyr Ser Ile Leu Leu Pro Val Leu Ile				
5130	5140	5150	5160	5170	
TTG CTA CTG GTT GGT TTG GTT TCG ATA TTT ATA GCG ACA AAT TTT GAT TAC	Leu Leu Leu Val Gly Leu Val Ser Ile Phe Ile Ala Thr Asn Phe Asp Tyr				
5180	5190	5200	5210	5220	
CCA ARG AAT CTT GTG CAA GTC ATG TCT CAG CAA GTA CTA TGG ATT TTT CTA	Pro Lys Asn Leu Val Gln Val Met Ser Gln Gln Val Leu Trp Ile Phe Leu				
5230	5240	5250	5260	5270	
GGT TCA GTA CTT GCT TTT GCT ATG TTA TTT AAT ACA GAG TTT CTT TGG	Gly Ser Val Leu Ala Phe Val Val Met Leu Phe Asn Thr Glu Phe Leu Trp				
5280	5290	5300	5310	5320	
AAG GTA ACC CCC TGG CTC TAT ATT TTC GGT CTG GTG CTT ATG GTT CTA CCT	Lys Val Thr Pro Trp Leu Tyr Ile Phe Gly Leu Val Leu Met Val Leu Pro				
5330	5340	5350	5360	5370	5380
CTC GTT TTC TAT TCT CCT GCT TTG GTA GCC TCG ACG GGA GCC AAA AAC TGG	Leu Val Phe Tyr Ser Pro Ala Leu Val Ala Ser Thr Gly Ala Lys Asn Trp				
5390	5400	5410	5420	5430	
GTA AGC ATT GGA TCT GCG CCC GAA TAA GCC TCAAGCTGCA TATTGATAG TCTG	Val Ser Ile Gly Ser Ala Pro Glu End				
5440	5450				
GGGTAA CCATCATCGA GGATC					

Fig. 2. Nucleotide sequences and deduced amino-acid sequences of *gtfK* and *orf1*. Potential ribosome binding sites (RBS) and -10 and -35 promoter elements are indicated.

and the mutants group. This indicated that in the absence of inter-species gene transfer, the existence of multiple GTFs might pre-date the divergence of the mutants group from *S. salivarius*.

Evidence for horizontal gene transfer

Partial recombination events will result in different parts

Fig. 5. Multiple alignment of the central portion of the deduced amino acid sequences of the catalytic domains of the eight available Gtfs. The constraints used in constructing the alignment are described in the text. The main blocks (BLOCK 1–BLOCK 8) as well as the three sub-blocks (BLOCK A–BLOCK C) used in subsequent phylogenetic analyses are shown. The residues aligned are: GtfJ, 180–1108; GtfK, 176–1078; GtfB, 166–1037; GtfC, 191–1075; GtfI (dn), 168–1046; GtfI (sb), 162–1035; GtfS, 156–1026; GtfD, 166–1069.

somewhat unlikely. Fourthly, in the overall phylogenetic tree (Fig. 6) GtfB and GtfC formed a significant group with the two GtfIs. However, in sub-tree 6, both GtfB and GtfC no longer formed a significant group with the two GtfI sequences but instead formed a grouping with GtfD with borderline significance. This suggested that both GtfB and GtfC contain sequences that originated from GtfD, implying a small partial recombination between *gtfD* and the precursor of *gtfB/gtfC*. Again this was plausible because *gtfD* is found in the same species as *gtfB* and *gtfC*. Lastly, in no case did GtfS, the Gtfs or GtfJ and GtfK show any sign of forming significant

Fig. 6. Unrooted phylogenetic tree constructed from the alignment in Fig. 5 using the neighbour-joining method (Saitou & Nei, 1987). Relative phylogenetic distances are indicated both numerically and by the length of the connecting lines.

groupings in the sub-trees (Fig. 7) that did not exist in the overall phylogenetic tree (Fig. 6).

Silent-site divergence analysis

The possibility of partial recombination events between *gtfB* and *gtfC* and between *gtfJ* and *gtfK* as investigated further using the replacement-site and silent-site divergence algorithms of Perler *et al.* (1980). This strategy depends on the principle that nucleotide identities at codon 'wobble' positions (or silent sites) are not constrained by the functional requirements of the gene product, so if two related but un-recombined genes are compared, they will exhibit a relatively constant level of silent-site divergence over their entire lengths. On the other hand, a recent partial recombination event will result in localized changes in the level of silent-site divergence. These analyses were carried out by determining the nucleotide sequences for blocks of pairwise aligned amino acid sequences within the entire catalytic domain and measuring the divergence of these nucleotide sequences at silent sites and replacement sites. Where possible, the blocks were arbitrarily made 99 nucleotides in length. However, the presence of gaps in the alignments necessitated some flexibility in block size, giving rise to blocks of between 60 and 150 nucleotides in length. In the case of *gtfB* and *gtfC*, it was clear that the divergence at silent sites was very high at the beginning of the genes, but then decreased significantly around nucleotide 1800 (Fig. 8a), strongly supporting the contention that these two genes had undergone a partial recombination. In the case of *gtfJ* and *gtfK* (Fig. 8b), the situation was different. There was no clear evidence of conservation at silent sites, although there may have been slight conservation in the region between residues

500 and 1000. This region coincides with the first of the sub-trees subjected to phylogenetic analysis, in which GtfJ and GtfK formed a highly significant group. Thus while there was some internally consistent evidence for partial recombination between *gtfJ* and *gtfK* it was far from clear-cut, indicating that such an event, if it had taken place at all, had not happened recently.

A similar approach was applied to the comparison of the two *gtfI* sequences, although in this case the very close relationship between the GtfIs meant that the nucleotide sequences could be readily aligned and much larger blocks of sequence used directly as input into the 'Diverge' program. It is clear from this analysis that there were dramatic differences in the silent-site divergence along the length of the two genes (Table 2). This observation supported the notion that there had been a partial recombination between these two sequences, despite their isolation from different bacterial species with different hosts. Also of interest was the large difference between the values of the silent-site and replacement-site divergences despite the high degree of sequence similarity. This would seem to indicate that the majority of the residues within these two enzymes can only undergo a small number of possible changes in order for these changes to remain selectively neutral.

Lastly, this approach was applied to a comparison of GtfD with GtfB and GtfC. In practice, GtfB was excluded from this analysis because of the almost perfect identity of GtfB and GtfC in the analysed region, and also because of a localized reading frame discontinuity in the alignment of GtfB with the other sequences observed

Table 2. Silent-site and replacement-site divergence within the catalytic domains of the GtfIs of *S. downei* and *S. sobrinus*

Nucleotides from start of coding region	Corrected percentage divergence	
	Silent sites	Replacement sites
1-248	69.4	8.0
267-1835	64.0	3.9
1836-2987	3.2	0.2

Table 3. Silent-site and replacement-site divergence within blocks A, B and C of the GtfC and GtfD of *S. mutans*

Blocks of alignment (see Fig. 5)	Corrected percentage divergence	
	Silent sites	Replacement sites
Block A	231.0	37.9
Block B	194.1	22.3
Block C	170.2	43.0

Fig. 7. Phylogenetic 'sub-trees' 1–8 (*a–h*) derived from blocks 1–8, respectively, of the alignment in Fig. 5. Bootstrapping confidence limits over 90 % are shown.

during the course of this analysis. [This discontinuity encompasses *GtfB* nucleotides 2397–2450 (numbered from the start codon) and may be explained by either a very recent single nucleotide deletion/insertion event in *gtfB*, or an error in the reported sequence of *gtfB*.] Furthermore, because of the overall relatively high level of divergence of *GtfD* from *GtfB*/*GtfC*, the analysis was confined to the region of interest highlighted by sub-tree analysis of block 6 (Fig. 7*f*). Three blocks of 150 nucleotides were considered. The central block (block B, Fig. 5) was judged to encompass the amino acid sequences that resulted in the grouping of *GtfD* with *GtfB* and *GtfC* (Fig. 7*f*), while the two other blocks (block A and block C, Fig. 5) occurred immediately before and after block B and were included for comparison. It was evident from the levels of silent-site

divergence in all three blocks that there was no sign of any conservation at silent sites; in fact the analyses indicated a saturating level of substitutions in all three blocks (Table 3). Consequently, if recombination between *GtfD* and the precursor of *GtfB* and *GtfC* had occurred in this region, it was too long ago for conservation at silent sites to be preserved.

Lack of evidence for inter-species transfer of intact gtf genes

The most common evidence presented for interspecies gene transfer is the presence of closely related genes in unrelated backgrounds, sometimes supported by base composition studies. However, in the case of the *gtf* genes, all the species are fairly closely related and have

Fig. 8. Divergences of *gtfB* and *gtfC* (a) and *gtfJ* and *gtfK* (b) along the coding sequences for their catalytic domains. Continuous lines represent silent site divergence while dashed lines represent replacement site divergence. Corrected percentage divergences were calculated as described by Perler *et al.* (1980).

similar base compositions. Therefore, the only approach open to us was to compare the tree in Fig. 6 with the phylogenies derived by DNA hybridization and rRNA sequencing (Schleifer *et al.*, 1984; Ludwig *et al.*, 1985; Schleifer & Kilpper-Balz, 1987; Bentley, 1991). These latter analyses have consistently found that the mutans group streptococci form a phylogenetically valid (albeit somewhat loose) grouping that does not include *S. salivarius*, that the extent of divergence within the mutans group is not greatly different from the divergence between the mutans group and *S. salivarius*, and that there is a specific close relationship between *S. downei* and *S. sobrinus*. If it were assumed that GtfB, GtfC, and the two GtfIs are all homologues in the strictest sense (i.e. are the products of the same gene duplication/species divergence events), and one does not consider GtfS and GtfD, then the phylogenies derived from DNA hybridization and rRNA sequences are fully consistent with the Gtf phylogenetic tree in Fig. 6. Specifically, the GTF-I enzymes from *S. mutans* and *S. downei/S. sobrinus* form a fairly loose but valid group that does not include sequences of *S. salivarius* origin, and the GtfIs from *S. downei* and *S. sobrinus* clearly cluster together. Since GtfS and GtfD do not form a group they remain something of a puzzle. It may be that they are not strict homologues and therefore may be the products of

different duplication events, having diverged prior to *S. mutans* and *S. downei* becoming separate species. Alternatively, there may be somewhat different selection pressures on GTF-S enzymes, resulting in faster divergence. Whatever the interpretation, there are no groupings in the Gtf phylogenetic tree that do not exist in the rRNA tree, implying that there is no evidence for the transfer of intact *gtf* genes between the species so far studied.

Discussion

The sequencing of *gtfK* completes the sequence analysis of the *gtfJK* locus as reported by Giffard *et al.* (1991). The arrangement and spacing of the genes is reminiscent of the *gtfB* and *gtfC* of *S. mutans* GS-5, although the gene products have somewhat different functions. Whereas both GtfB and GtfC have been reported to be primer-independent enzymes that synthesize a mixture of soluble and insoluble glucans (Aoki *et al.*, 1986; Hanada & Kuramitsu, 1988), GtfJ and GtfK are both primer-dependent enzymes that synthesize insoluble and soluble glucans respectively (Pitty *et al.*, 1989). Thus, the activity of GtfJ most resembles that of the GtfIs of *S. downei* and *S. sobrinus* while that of GtfK is similar to the GtfD of *S. mutans*.

GtfK can be clearly aligned with all other Gtf sequences, and contains the putative signal sequence and C-terminal repeats that are typical of this family of enzymes. As with the other Gtfs, the most abundant repeated sequence is the 'A' repeat while five of these 'A' repeats are followed by another class of repeat that shows similarity to the 'D' repeats of GtfJ and therefore has been classified as such. However, the classification of these repeats represents something of a problem in our view, as all of them show a certain level of similarity with one another as well as to the repeats in the ligand-binding domains of the toxins from *Clostridium difficile* and in the lysins from *Streptococcus pneumoniae* (Wren, 1991), which are classified according to a completely different scheme. The possibility of a common underlying structure to all these repeat classes is currently under investigation.

Our evolutionary analysis of the eight *gtf* genes so far sequenced was based on constructing a phylogenetic tree from a highly homologous region in the catalytic domain of these enzymes and then attempting to falsify it by searching for evidence of recombination and/or horizontal gene transfer. The lengths of the sequences aligned meant that a phylogenetic tree with extremely good confidence limits could be constructed. This analysis showed that enzymes with similar activities did not group together and that the existence of multiple Gtfs

may have pre-dated the divergence of *S. salivarius* from the mutans group.

Partial recombination events were tested for by seeking out localized deviations from the overall relationships between the sequences by constructing a series of sub-trees from portions of the alignment and also by carrying out a series of silent-site divergence measurements. From these analyses, clear corroborative evidence was obtained for the partial recombination between *gtfB* and *gtfC*, and also between the *gtfI* genes of *S. downei* and *S. sobrinus*. Recombination between *gtfB* and *gtfC* was not surprising as these two genes lie next to each other on the *S. mutans* GS-5 chromosome. Furthermore, polymorphisms at this locus have been documented, indicating the potential for rearrangements between these two genes (Chia *et al.*, 1991; Yamashita *et al.*, 1992). In contrast, however, the apparent partial recombination between the two *gtfI* genes was unexpected. These genes are not only found in different species of streptococci, but the streptococci themselves have different hosts. Gene transfer between the two species would therefore appear at first sight to be unlikely. It may simply be that the two species live in both monkeys and man. Whatever the explanation, these recombination events imply that the distances between *GtfB* and *GtfC* and between the two *GtfIs* in Fig. 6 are an underestimation of the true phylogenetic distances.

The evidence for recombination between the precursor of *gtfB* and *gtfC* with *gtfD* and also for recombination between *gtfJ* and *gtfK* was much less convincing because it could not be corroborated by silent-site divergence analysis. However, because each group of genes is resident on the chromosome of a specific species, recombination events would not be unexpected. Thus while recombination is probable it remains unconfirmed, and so the divergence between the sequences can be thought of as a minimum not a maximum relative to the time since the sequences diverged.

Apart from the situation with the two *gtfI* genes, it was difficult to discern any evidence for inter-species gene transfer. However, such events are very difficult to rule out. GTF enzymes are by no means ubiquitous in the genus *Streptococcus*, yet rRNA sequence studies (Bentley *et al.*, 1991) show no evidence that the mutans group and *S. salivarius* form any sort of natural group. As both species contain *gtf* genes it is plausible that an ancestral *gtf* gene(s) was transferred from the mutans group common ancestor to the *S. salivarius* ancestor or vice versa. However, it is not necessary to invoke such an event to rationalize the rRNA phylogenetic tree with the *Gtf* phylogenetic tree since the *Gtf* divergence evident both within the mutans group and within *S. salivarius* suggests that such an event must be very ancient if it happened at all. The unravelling of these early events must await a more sophisticated analysis.

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