Genetic Exchange Between Oral Streptococci During Mixed Growth

By HOWARD K. KURAMITSU* AND VALERIE TRAPA

Department of Microbiology-Immunology, Northwestern University Medical-Dental Schools,
Chicago, IL 60611, USA

(Received 3 May 1984)

To determine whether oral streptococci might exchange genetic information in the oral cavity, paired transformable strains of *Streptococcus mutans*, *Streptococcus sanguis* and *Streptococcus milleri* were grown together. Chromosomal and plasmid-borne antibiotic resistance markers could be readily transferred from *S. mutans* GS-5 to *S. milleri* NCTC 10707 or *S. sanguis* Challis during mixed growth. However, no exchange from the latter two organisms to strain GS-5 could be detected under these conditions. The transfer of genetic information from *S. sanguis* to *S. milleri* was also observed.

INTRODUCTION

Human dental caries results from the acidogenic properties of micro-organisms present in dental plaque (Gibbons & van Houte, 1975). Although primary attention has been focused on *Streptococcus mutans* in cariogenesis, other oral plaque streptococci such as *Streptococcus sanguis* or *Streptococcus milleri* may also contribute to this disease process. As an adjunct to biochemical studies of these micro-organisms, more recent investigations have emphasized genetic approaches (Robeson *et al.*, 1983; Perry *et al.*, 1983), and certain human oral isolates of *S. mutans* (Perry & Kuramitsu, 1981), *S. sanguis* and *S. milleri* (Colman, 1969) have been transformed with homologous or heterologous DNA.

Since transformation of bacteria has been demonstrated after infection into animal hosts (Ottolenghi & MacLeod, 1963), it is of interest to determine whether DNA could be exchanged between streptococci in the oral cavity. Chromosomal genetic markers have been exchanged between transformable strains of *S. mutans*, *S. sanguis* and *S. milleri* *in vitro* (Colman, 1969; Westergren & Emilson, 1977; Perry & Kuramitsu, 1981). We have developed a model system to determine whether genetic exchange could occur between oral streptococci during growth in mixed culture. In this paper, the results from this system are discussed in regard to possible genetic exchange in the human oral cavity.

METHODS

Organisms. The human dental plaque isolate *S. mutans* GS-5 (serotype c) was maintained and routinely grown in Todd-Hewitt (Difco) medium as previously described (Kuramitsu, 1973). *S. milleri* NCTC 10707 isolated from the human oral cavity was obtained from the National Collection of Type Cultures (London, UK) and *S. sanguis* Challis, originally isolated from human serum, from D. Perry (Northwestern University, Chicago, Ill., USA). All cultures were routinely transferred weekly into Todd-Hewitt medium. *S. sanguis* V736 containing the erythromycin-resistance plasmid pVA736 was obtained from F. L. Macrina (Virginia Commonwealth University, Richmond, Va., USA) and was maintained in Todd-Hewitt medium containing erythromycin ($10\ \mu\text{g ml}^{-1}$).

Antibiotic-resistant organisms. Mutants of the oral streptococci containing chromosomal streptomycin (Str) or rifampicin (Rif) resistance markers were isolated following nitrosoguanidine mutagenesis (Adelberg *et al.*, 1965). Exponential-phase cells of each organism were mutagenized with nitrosoguanidine ($100\ \mu\text{g ml}^{-1}$) for 30 min at 37°C , washed with sterile saline to remove the mutagen, and grown in Todd-Hewitt broth for 18 h at 37°C . Samples of the cultures were then grown on Trypticase soy agar (Difco) plates in the anaerobic GasPak System (BBL) containing either streptomycin ($200\ \mu\text{g ml}^{-1}$) or rifampicin ($10\ \mu\text{g ml}^{-1}$). Each isolate was purified by restreaking and re-examined for transformability before being used in the mixed growth experiments.

Plasmid pVA736, coding for erythromycin resistance (Ery^r), was isolated from *S. sanguis* V736 as previously described (Macrina *et al.*, 1980). The purified plasmid was then introduced into both *S. mutans* GS-5 and *S. milleri* NCTC 10707 by transformation (Kuramitsu & Long, 1982). The presence of plasmid pVA736 in the resultant transformants was verified following agarose gel electrophoresis of cleared lysates (Kuramitsu & Long, 1982).

Transformation during mixed growth. Freshly grown inocula of each organism, approximately 8×10^6 c.f.u., were incubated separately and together in Todd-Hewitt broth (5 ml) for 18 h at 37 °C. Samples (0.1 ml) were then grown anaerobically for 48 h at 37 °C on agar plates containing appropriate antibiotics to select for transfer of antibiotic resistance. Initial experiments indicated that 18 h incubation yielded approximately optimal yields of transformants during mixed growth. Differentiation between *S. mutans* and either *S. sanguis* or *S. milleri* in mixed culture was accomplished by using Mitis salivarius agar (Difco) plates. When *S. milleri* and *S. sanguis* were grown together, colonies were differentiated on the basis of the relative sensitivities of the two strains to the bacteriocin produced by *S. mutans* GS-5 (*S. sanguis* Challis, but not *S. milleri* NCTC 10707, is sensitive to this bacteriocin). Isolated colonies were tested for bacteriocin sensitivity as described previously, by the soft agar overlay method (Paul & Slade, 1975).

The frequency of transformation for each organism during mixed growth was calculated as the number of double antibiotic resistant mutants of each organism divided by the total number of each organism. Spontaneous mutation frequencies to antibiotic resistance were determined on antibiotic selection plates. Duplicate plates were prepared for each sample and the values represent the average of each pair. The data presented represent typical results from experiments repeated two to four times.

RESULTS

Chromosome exchange between S. mutans and S. milleri

To determine whether oral streptococci might exchange gene fragments by natural transformation during mixed growth, *S. mutans* GS-5, *S. sanguis* Challis, and *S. milleri* NCTC 10707 were selected as representative transformable human isolates of each species and grown in mixed culture. It was anticipated that some of the cells would lyse following growth in culture and release chromosomal DNA fragments which might subsequently transform heterologous cells in the same culture. In one group of experiments, *S. mutans* GS-5 (Rif^r) was grown with *S. milleri* NCTC 10707 (Str^r) and the production of Rif^r Str^r *S. milleri* and *S. mutans* cells was determined. The Rif^r phenotype from *S. mutans* was transformed into *S. milleri* during mixed growth (Table 1). The transformation frequency observed (7.5×10^{-6}) was almost two orders of magnitude higher than the spontaneous mutation rate of *S. milleri* (Str^r) to Rif^r (9.2×10^{-8}). The involvement of transformation was indicated by the complete elimination of double antibiotic-resistant mutants when DNAase was present during growth. In contrast to the results with *S. milleri*, no Str^r Rif^r *S. mutans* GS-5 colonies were detected. The lack of detectable transformation of strain GS-5 grown in mixed culture was not limited to the Rif^r strain since subsequent results (not shown) also demonstrated that the Str^r marker could not be transferred from *S. milleri* to another GS-5 strain containing plasmid pVA736.

Genetic exchange between S. mutans and S. sanguis

Since *S. sanguis* Challis has previously demonstrated relatively high *in vitro* transformation frequencies with chromosomal DNA from *S. mutans* GS-5 (Perry & Kuramitsu, 1981), it was anticipated that streptococcal chromosomal markers might be readily transformed into these cells in mixed culture. Indeed, the Rif^r marker from *S. mutans* GS-5 was transformed into *S. sanguis* Challis harbouring plasmid pVA736 during mixed growth (Table 2). However, no transfer of plasmid pVA736 from *S. sanguis* Challis into *S. mutans* GS-5 could be detected. Likewise, in a similar experiment the Str^r marker from *S. sanguis* Challis could not be transferred to *S. mutans* GS-5 during mixed growth (data not shown).

Chromosomal transfer between S. sanguis and S. milleri

Since *S. sanguis* Challis (Perry & Kuramitsu, 1981) and *S. milleri* NCTC 10707 (Colman, 1969) exhibit relatively high *in vitro* transformation frequencies and both act as transformation recipients in mixed culture with *S. mutans* GS-5 (Tables 1 and 2), it was of interest to determine whether each acts as a recipient when the strains were grown together. When *S. milleri* (Str^r) was grown with *S. sanguis* (Rif^r), Str^r Rif^r transformants of *S. milleri* were isolated at a frequency of 2×10^{-5} , but no Str^r Rif^r *S. sanguis* transformants could be detected following growth.

Table 1. Chromosomal transformation between *S. mutans* and *S. milleri* in mixed culture

S. mutans GS-5 (Rif^r) and *S. milleri* NCTC 10707 (Str^r) were grown individually and together, as indicated, for 18 h at 37 °C and mixed transformation was assessed as described in Methods. One mixed culture contained filter-sterilized DNAase (24 µg ml⁻¹) throughout the incubation period. Rifampicin (10 µg ml⁻¹) and streptomycin (200 µg ml⁻¹) were added to appropriate agar plates.

Bacteria	Frequency of conversion of*	
	<i>S. mutans</i> to Str ^r	<i>S. milleri</i> to Rif ^r
<i>S. mutans</i> GS-5 (Rif ^r)	$< 7.2 \times 10^{-8}$	—
<i>S. mutans</i> GS-5 (Rif ^r) + <i>S. milleri</i> NCTC 10707 (Str ^r)	$< 2.1 \times 10^{-8}$	7.5×10^{-6}
<i>S. mutans</i> GS-5 (Rif ^r) + <i>S. milleri</i> NCTC 10707 (Str ^r) + DNAase	$< 2.1 \times 10^{-8}$	$< 6.0 \times 10^{-7}$
<i>S. milleri</i> NCTC 10707 (Str ^r)	—	9.2×10^{-8}

* The frequency of conversion refers to spontaneous mutation rates for individual organisms, or transformation rates in mixed cultures.

Table 2. Genetic exchange between *S. sanguis* and *S. mutans* in mixed culture

S. mutans GS-5 (Rif^r) and *S. sanguis* Challis (pVA736) were grown together under standard conditions and treated as described in Methods using Mitis salivarius agar plates containing rifampicin (10 µg ml⁻¹) and erythromycin (10 µg ml⁻¹).

Bacteria	Frequency of conversion of*	
	<i>S. mutans</i> to Ery ^r	<i>S. sanguis</i> to Rif ^r
<i>S. mutans</i> GS-5 (Rif ^r)	$< 7.8 \times 10^{-8}$	—
<i>S. mutans</i> GS-5 (Rif ^r) + <i>S. sanguis</i> Challis (pVA736)	$< 5.3 \times 10^{-7}$	3.6×10^{-6}
<i>S. sanguis</i> Challis (pVA736)	—	9.7×10^{-8}

Plasmid exchange between oral streptococci

Since cryptic plasmids have been demonstrated in some strains of oral streptococci (Macrina *et al.*, 1977), it was of interest to determine whether plasmid transformation could be demonstrated in mixed culture. The Ery^r-coding plasmid pVA736 was chosen as a readily detectable indicator of plasmid exchange. This plasmid is composed of the naturally occurring *Streptococcus ferus* cryptic plasmid pVA380-1 ligated with a DNA fragment coding for Ery^r isolated from another streptococcal plasmid, pVA1 (Macrina *et al.*, 1980). Plasmid pVA736 can be readily transformed *in vitro* into *S. sanguis* Challis (Macrina *et al.*, 1980), *S. mutans* GS-5 (Kuramitsu & Long, 1982), and *S. milleri* NCTC 10707 (H. K. Kuramitsu, unpublished results). When *S. mutans* GS-5 (pVA736) was grown with *S. milleri* (Str^r), transformation of the latter organism to Ery^r was detected at a frequency of 5.8×10^{-5} . No transformation of *S. mutans* GS-5 with the Str^r chromosomal marker of *S. milleri* could be demonstrated in these experiments. Likewise, when *S. mutans* GS-5 containing pVA736 was grown with *S. sanguis* Challis, the plasmid was transferred at a frequency of 4.5×10^{-7} . In contrast, plasmid pVA736 could not be transferred to *S. mutans* GS-5 in mixed culture from either *S. milleri* (data not shown) or *S. sanguis* (Table 2). As with the chromosomal markers, plasmid pVA736 could also be transferred from *S. sanguis* Challis to *S. milleri* NCTC 10707 in mixed culture at a frequency of 2×10^{-6} , while no transfer in the reverse direction could be detected (data not shown).

DISCUSSION

The results of the present study indicate that no transformation of *S. mutans* GS-5 with chromosomal or plasmid-derived antibiotic resistance markers from either *S. sanguis* Challis or *S. milleri* NCTC 10707 could be detected in mixed culture. However, both plasmid and chromosomal DNA derived from either organism can readily transform strain GS-5 when

added in the standard *in vitro* transformation system (Perry & Kuramitsu, 1981; Kuramitsu & Long, 1982; H. K. Kuramitsu, unpublished results). The inability to demonstrate transformation of strain GS-5 is unlikely to be due to restriction or sequence specificity since no transformation could be detected when two GS-5 strains (GS-5 Str^r and GS-5 Rif^r) were grown together. Transformation of strain GS-5 depends on the development of competence, which is strictly dependent upon culture conditions as well as on the presence of serum (Perry & Kuramitsu, 1981). It is therefore likely that the mixed culture environment we used was unsuitable for the development of competence in strain GS-5. In addition, the inability to transform GS-5 in mixed culture does not result from the antagonistic effects of either *S. sanguis* Challis or *S. milleri* NCTC 10707 since no inhibitory effects of the latter organisms on strain GS-5 could be detected. Therefore, these results suggest that little or no transformation of *S. mutans* strains may occur in the human oral cavity.

In contrast to *S. mutans* GS-5, both plasmid and chromosomal transformation of *S. sanguis* Challis and *S. milleri* NCTC 10707 could be demonstrated in mixed cultures. Apparently the higher transformation frequencies demonstrated for these organisms under optimal conditions are sufficient to allow detection of transformation in mixed culture despite the non-ideal transformation conditions. If strains Challis and NCTC 10707 are representative of some strains of *S. sanguis* and *S. milleri* present in the human oral cavity, it is possible that some members of these species may undergo natural transformation in this habitat. However, since the model system of the present investigation is clearly distinct from that found in the oral cavity, implantation experiments in animal models would be necessary before the results reported here could be extrapolated to the situation *in vivo*.

We gratefully acknowledge the discussion and advice of D. Perry and F. L. Macrina during the course of this investigation. This study was supported by Public Health Service grant DE-03258 from the National Institute of Dental Research.

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