Proc. Natl. Acad. Sci. USA
Vol. 75, No. 7, pp. 3484–3487, July 1978
Microbiology

"Conjugal" transfer of plasmid DNA among oral streptococci

(*Streptococcus mutans*/*Streptococcus salivarius*/*Streptococcus sanguis*/ antibiotic resistance)

DONALD J. LEBLANC, ROBERT J. HAWLEY*, LINDA N. LEE, AND EDWARD J. ST. MARTIN

Laboratory of Microbiology and Immunology, National Institute of Dental Research, Bethesda, Maryland 20014

Communicated by DeWitt Stetten, Jr., April 18, 1978

ABSTRACT **The β plasmid from *Streptococcus faecalis* strain DS5, which codes for resistance to erythromycin and lincomycin, was introduced into a Lancefield group F streptococcus, strain DR1501, by transformation. This strain, DR1501 (β), was found to be an excellent donor of the β plasmid and readily transferred the resistance markers to various lactic acid bacteria, including certain strains of *S. mutans*, *S. sanguis*, and *S. salivarius*. Evidence is presented indicating that the transfer of the β plasmid is mediated by a mechanism that requires cell-to-cell contact. The transfer of plasmid DNA during conjugation has been confirmed by the isolation of β plasmid from transconjugant clones and by their ability to then serve as donors of the erythromycin and lincomycin resistance markers.**

The transfer of genetic material by conjugation has been described for numerous genera of Gram-negative bacteria (1), but this mechanism of gene transfer is largely unknown among Gram-positive bacteria. The transmission of genetic material by cell-to-cell contact in a Gram-positive bacterium was first suggested by Raycroft and Zimmerman (2) working with a strain of *Streptococcus faecalis*. Plasmid-mediated gene transfer in this species was actually confirmed by Jacob and Hobbs (3). Several reports have now shown that a number of *S. faecalis* plasmids are capable of self-transfer (4–6) as well as mobilization of other plasmids and chromosomal genes (5, 7, 8).

Van Embden *et al.* (6) showed that two group D streptococci were able to transfer erythromycin (Em) resistance to a strain of group B streptococcus. Recently, V. Hershfield (personal communication) showed that the Em resistance β plasmid from *S. faecalis* strain DS5 (9) is transmissible and capable of mediating its own transfer to strains of groups B and D streptococci. In this communication we report the transfer of β plasmid from a group F streptococcus to three species of oral streptococci, *S. mutans*, *S. sanguis*, and *S. salivarius*, when donor and recipient cells were incubated together on a membrane filter. *S. sanguis* and *S. mutans* transconjugants were shown to have acquired a plasmid with physical properties identical to those of the β plasmid. The presence of the β plasmid in *S. mutans* confers upon this organism the ability to serve as a β plasmid donor in intraspecies matings.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. All relevant characteristics of bacterial strains used in this study are listed in Table 1. The plasmid pAMβ1 (5) is here referred to as β plasmid. Three types of media were used for cell growth: complex medium (CM), defined medium (DM), and fluid thioglycolate broth (FTB), all of which have been described (10). CM was supplemented with 20 mM D-glucose, and DM with 30 mM D-mannitol. Final concentrations of antibiotics were 25 μg/ml for Em, lincomycin (Lm), and tetracycline (Tc) and 2 mg/ml for streptomycin (Sm). Solid media contained 1.5% agar (Difco). Incubation was at 37° in all cases.

Mating Conditions. Stock cultures, in FTB, of donor and recipient strains were diluted 1:10 to 1:20 in FTB and incubated overnight. For donor cultures the FTB was supplemented with Em to ensure maintenance of the β plasmid. Overnight cultures were diluted 1:10 in CM + glucose (no antibiotic) and allowed to grow to mid-exponential phase (~5×10^8 colony-forming units/ml). Donor and recipient cultures (0.5 ml of each) were mixed and collected on sterile membrane filters (type HA; pore size, 0.45 μm, Millipore) under vacuum. Filters were then transferred to CM + glucose plates and incubated overnight in an H_2/CO_2 atmosphere (GasPak, BBL). Control filters, containing donor or recipient cells alone, were included in all experiments.

After overnight incubation, the filters were transferred to 5 ml of CM + glucose and the cells were dispersed by agitation (Vortex Genie, maximum speed). The cells were allowed to reach mid-exponential phase and then dilutions were spread on appropriate media for selection of transconjugants. Transfer of the β plasmid to *S. sanguis* (DR0502) and *S. salivarius* (DR0251) was determined by selection on CM + glucose plates containing Em and Tc. Selection of transconjugants from mating mixtures containing the group F donor [DR1501 (β)] and *S. mutans* (DR0001) was on DM + mannitol plates containing Em. Sm was added to the latter to select for plasmid transfer from strain DR0001 (β) to strain DR0001/1.

Concentrations of donor and recipient cells in the mating mixtures were determined on the following agar plates: (*i*) DR1501 (β) on CM + glucose + Em; (*ii*) DR0502 and DR0251 on CM + glucose + Tc; (*iii*) DR0001 on DM + mannitol; (*iv*) DR0001 (β) on DM + mannitol + Em; and (*v*) DR0001/1 on DM + mannitol + Sm. All plates were incubated for 2–4 days under anaerobic conditions (GasPak, BBL).

Labeling of DNA and Preparation of Cell Lysates. DNA was labeled by growing cells for 16 hr in 100 ml of CM + glucose supplemented with [^{3}H]thymidine (7.0 μCi/ml; 40–50 Ci/mmol; New England Nuclear Corp.) and 10 mM L-threonine. Em was also included when the strain was resistant to this antibiotic. Cells were lysed with sodium dodecyl sulfate as described (11).

Isolation of Plasmid DNA. Plasmid DNA was separated from the bulk of chromosomal DNA by a procedure adapted from Currier and Nester (12) as described (11). Covalently closed circular (CCC) plasmid DNA was separated from open circular molecules and residual chromosomal DNA in equi-

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: Em, erythromycin; Lm, lincomycin; Sm, streptomycin; Tc, tetracycline; CCC, covalently closed circular; β, plasmid pAMβ1; CM, complex medium; DM, defined medium; FTB, fluid thioglycolate broth.

* On leave from the Department of Microbiology, Georgetown University Schools of Medicine and Dentistry, 3900 Reservoir Road, Washington, DC 20007.

Table 1. Bacterial strains used in this study

Strain	Species	Relevant characteristics*	Origin
DR1501(β)	Group F	Em^R, Lm^R, Tc^S, mannitol$^-$, sorbitol$^-$, lactose$^-$, raffinose$^-$, inulin$^-$, β hemolysis†	Formerly DL8-12 (15)
DR0502	*S. sanguis* (type I)	Em^S, Lm^S, Tc^R, raffinose$^+$, inulin$^+$, α hemolysis†	Clinical isolate
DR0251	*S. salivarius*	Em^S, Lm^S, Tc^R, lactose$^+$, inulin$^+$	Clinical isolate
DR0001	*S. mutans*	Em^S, Lm^S, Sm^S, mannitol$^+$, sorbitol$^+$, lactose$^+$	Formerly 6715-10 from NIDR collection
DR0001/1	*S. mutans*	Same as DR0001, but Sm^R	Spontaneous Sm^R (2 mg/ml) of strain DR0001

* R, resistant; S, susceptible
† Hemolysis was determined on Columbia agar base medium containing 5% sheep blood.

librium density gradients containing ethidium bromide, as described (11).

Agarose Gel Electrophoresis. Fractions from dye buoyant density gradients containing CCC plasmid DNA were pooled and ethidium bromide was extracted with cesium chloride-saturated n-propanol. Cesium chloride was removed by dialysis against 0.01 M Tris·HCl/0.01 M EDTA/0.05 M NaCl, pH 8.2 (11). The dialyzed pools were concentrated by ethanol precipitation and resuspended in the same buffer. Plasmid molecular weights were estimated by electrophoresis in 0.7% agarose slab gels (13) in Tris-acetate buffer (pH 7.7) (14) at 60 mA, 80 V, for 4.5–5 hr. Gels were stained overnight in 2 liters of water containing 0.8 μg of ethidium bromide per ml. DNA bands were viewed with a short-wavelength lamp (Mineralight, Ultraviolet Products, Inc., San Gabriel, CA).

RESULTS

Conjugal Transfer of Em Resistance. The transfer of β plasmid DNA, by transformation, to a Lancefield group F streptococcus has been reported (15). All transformed isolates examined contained a plasmid physically identical to the β plasmid from *S. faecalis*, strain DS5. One of these isolates, strain DR1501 (β), was chosen as a potential β plasmid donor for conjugation experiments with various strains of oral streptococci. Strain DR1501 (β) is resistant to Em at >1 mg/ml and to Lm at 500 μg/ml. This strain is also sensitive to Tc at <1 μg/ml and is unable to grow at the expense of mannitol on DM. Several isolates of *S. sanguis*, *S. salivarius*, and *S. mitis*, from clinical sources, were chosen as potential recipients of the β plasmid. All of these strains were resistant to Tc at >25 μg/ml and sensitive to Em and Lm at <5 μg/ml. When these isolates were mixed with strain DR1501(β), one of five *S. sanguis*, two of four *S. salivarius*, and none of five *S. mitis* strains produced colonies on CM + glucose agar plates containing both Em and Tc. One laboratory strain, DR0001, but none of seven clinical isolates of *S. mutans*, produced colonies on DM + mannitol agar plates containing Em after incubation with strain DR1501 (β). In all of these experiments, neither donor nor recipient alone was able to produce colonies when up to 10^9 colony-forming units were spread on selective plates containing both antibiotics.

Table 2. Frequency of transfer of Em resistance from strain DR1501 (β) to oral streptococci

Recipient strain	Species	Transfer frequency*
DR0502	*S. sanguis*	1.5×10^{-4}
DR0251	*S. salivarius*	3.6×10^{-6}
DR0001	*S. mutans*	2.6×10^{-6}

* Frequencies are expressed as the number of Em-resistant recipient colonies observed per number of donor colony forming units present at the end of mating.

The frequencies of transfer of Em resistance from strain DR1501 (β) to three of the recipient strains tested are shown in Table 2.

The identities of transconjugant clones were confirmed by at least three additional properties that distinguished them from the donor, group F, strain (see Table 1). Transconjugants of strain DR0502 were able to utilize raffinose and inulin and were shown to be γ hemolytic on sheep blood agar. Strain DR0251 transconjugants were lactose$^+$ and inulin$^+$ and exhibited a colonial morphology, on CM + glucose agar, characteristic of *S. salivarius*. *S. mutans* transconjugants were further identified by their ability to utilize mannitol, sorbitol, and lactose.

Several control experiments were performed to determine the nature of the observed transfer. In one experiment, 0.5 ml of a sterile culture filtrate of strain DR1501 (β) was incubated with the recipient strain for 30–60 min prior to collection on a membrane filter. When such a cell-free filtrate was used instead of the donor culture, no transfer of Em resistance to either strain DR0502 or DR0251 was observed. These results suggested that the transfer of Em resistance obtained in the standard mating mixtures was not mediated by bacteriophage. Strains DR0502 and DR0251 were unable to produce colonies on plates containing Em when the DR1501 (β) donor was replaced with a DR1501 isolate that had been cured of the β plasmid. These results confirm the requirement for the β plasmid in this mating system.

The influence of DNase on transfer frequencies between strain DR1501 (β) and the *S. salivarius* and *S. sanguis* recipients is shown in Table 3. With the *S. salivarius* recipient, strain DR0251, the frequency appeared to be lowered by approximately 20% in the presence of 100 μg of DNase. However, $MgSO_4$ (0.02 M) alone also appeared to lower the frequency to approximately the same extent. When the mating mixture contained the *S. sanguis* strain (DR0502) as a recipient, the presence of DNase resulted in a 75% reduction in transfer frequency. This was almost 3 times greater than the reduction caused by $MgSO_4$ with this recipient. Therefore, the transfer of Em resistance from the donor strain to the *S. sanguis* recip-

Table 3. Effects of DNase and $MgSO_4$ on the frequency of plasmid transfer

Donor/recipient mixture	Component added	Frequency*
DR1501 (β)/DR0251 (group F)/(*S. salivarius*)	None	4.2×10^{-6}
	DNase + $MgSO_4$	3.5×10^{-6}
	$MgSO_4$	2.8×10^{-6}
DR1501 (β)/DR0502 (group F)/(*S. sanguis*)	None	2.0×10^{-4}
	DNase + $MgSO_4$	4.8×10^{-5}
	$MgSO_4$	1.3×10^{-4}

* Frequencies are expressed as indicated in Table 2.

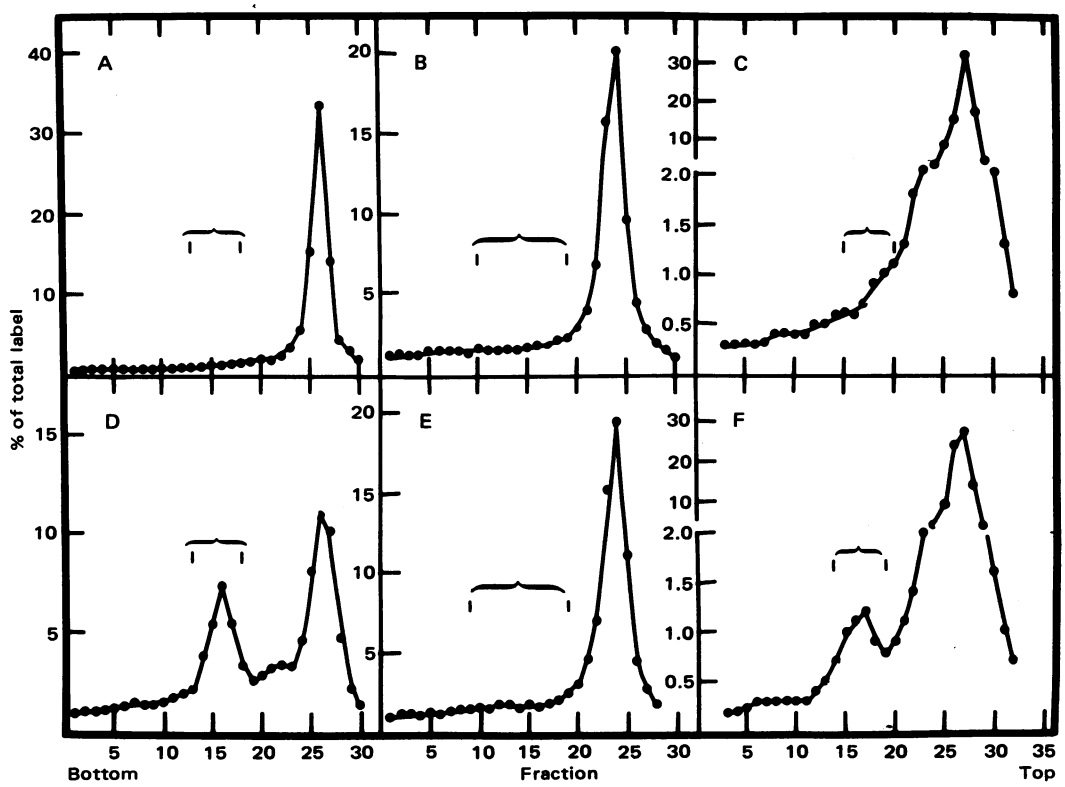

FIG. 1. Ethidium bromide/cesium chloride buoyant density gradient centrifugation of DNA from oral streptococcus recipients and Em-resistant transconjugants. Fractions were pooled as indicated. Strain analyzed and total ^3H cpm in gradient were as follows: (*A*) *S. sanguis* DR0502, 365,000; (*B*) *S. salivarius* DR0251, 200,000; (*C*) *S. mutans* DR0001, 283,000; (*D*) *S. sanguis* DR0502 (β), 945,000; (*E*) *S. salivarius* DR0251 (β), 268,000; (*F*) *S. mutans* DR0001 (β), 469,000.

ient may, in fact, be partially due to a spontaneous release of transforming DNA from the donor, although other explanations are possible. The results of these control experiments suggest that the transfer of Em resistance from strain DR1501 (β) to *S. salivarius* is due to a mechanism that is similar to conjugation and that the transfer to the *S. sanguis* recipient is also, at least in part, the result of conjugation. The reason for the effect of $MgSO_4$ is not clear.

Conjugal Transfer of the β Plasmid. The β plasmid codes for resistance to both Em and Lm. Evidence for the presence of this plasmid in transconjugant isolates was provided by the observation that Em-resistant transconjugants of all three species were also resistant to Lm at a concentration of at least 25 μg/ml. The presence of β plasmid DNA was confirmed by the physical isolation and partial characterization of plasmid DNA from transconjugants of two of the three species studied.

The DNA of strains DR0502, DR0251, and DR0001 and an Em-resistant transconjugant of each was labeled with [^3H]-thymidine and extracted. The results of dye buoyant density gradient analyses, illustrated in Fig. 1, suggest that these strains did not harbor any detectable plasmid molecules prior to mating with strain DR1501 (β). DNA from the Em-resistant transconjugants of strains DR0502 (*S. sanguis*) and DR0001 (*S. mutans*), produced a second band in these gradients, at an apparent buoyant density characteristic of the β plasmid from strain DR1501 (β). In three separate experiments, we were unable to obtain evidence for the presence of a CCC plasmid species in the *S. salivarius* transconjugant.

Fractions were pooled from that region of each gradient illustrated in Fig. 1 where a plasmid band was observed or would have been expected. The pools were prepared for and subjected to agarose gel electrophoresis. These results are shown in Fig. 2. Included in the gel was CCC β plasmid DNA from strain DR1501 (β) (lane J). The dye buoyant density gradient pools from strains DR0502, DR0251, and DR0001 failed to produce any detectable plasmid bands. However, strains DR0502 (β) and DR0001 (β) produced a major band migrating at approximately the same rate as the β plasmid from strain DR1501 (β). The estimated molecular weights were 16.8×10^6 for the plasmid from strain DR0502 (β) and 17×10^6 for the plasmids from strains DR0001 (β) and DR1501 (β). The minor, slower-migrating band in lanes B, H, and J corresponds to a nicked circular form of the β plasmid. As in Fig. 1, *S. salivarius*, strain DR0251 (β), produced no plasmid band in the agarose gel. Whether this reflects a rapid degradation of the plasmid in this strain after extraction or integration into the host chromosome has not been determined.

Further confirmation of the transfer of the β plasmid to these oral streptococci by strain DR1501 (β) was obtained with the *S. mutans* transconjugant. Strain DR0001 (β) could now serve as a donor of the β plasmid to a Sm-resistant mutant of the parent, strain DR0001/1. Transconjugants were selected on DM + mannitol plates containing both Em and Sm. As shown in Table 4, the frequency of transfer of the β plasmid between the two *S. mutans* strains was more than 50-fold higher than the frequency originally observed in the mating between the group F donor and the *S. mutans* recipient.

DISCUSSION

The data presented here provide evidence for a genetic transfer system, resembling conjugation, among three species of oral streptococci. Although these matings were quite reproducible by the membrane filter method described, attempts to effect transfer of the β plasmid in broth cultures were unsuccessful. DNase had virtually no effect on the frequency of transfer of

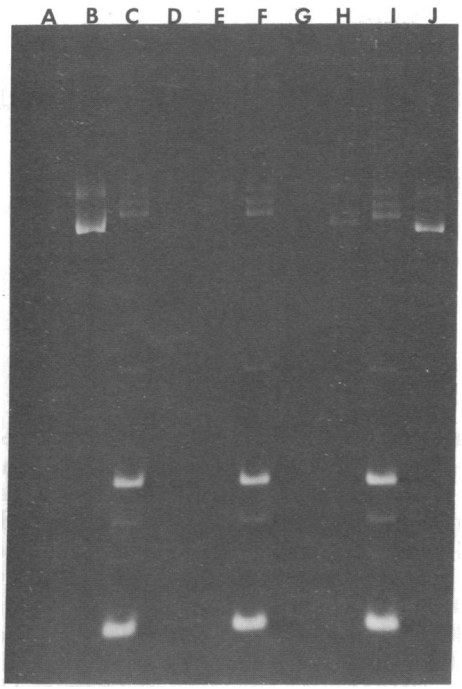

FIG. 2. Agarose gel electrophoresis of plasmid pools from Fig. 1. Strains and total ^3H cpm loaded were as follows: lane A, *S. sanguis* DR0502, 720; lane B, *S. sanguis* DR0502 (β), 1800; lane D, *S. salivarius* DR0251, 1200; lane E, *S. salivarius* DR0251 (β), 1280; lane G, *S. mutans* DR0001, 300; lane H, *S. mutans* DR0001 (β), 600; lane J, group F DR1501 (β), 14,000. Lanes C, F, and I contained approximately 500 ng of plasmid DNA from a strain of *S. lactis* harboring plasmid species of 28.5, 23.5, 29, 5.8, 3.1, and 1.5 megadaltons (unpublished data) for estimation of molecular weights (13).

the β plasmid to the *S. salivarius* recipient but appeared to decrease significantly the transfer frequency to the *S. sanguis* strain. The latter result suggests that, with this recipient strain, transformation may be partially responsible for the observed transfer. However, it is also possible that the contact between mating pairs is such that the DNA being transferred is, at some point, susceptible to the action of DNase.

After transfer to a new host, the β plasmid has been shown to retain the expression of both Em and Lm resistance as well as conjugative functions. In fact, the frequency of the intraspecies transfer *S. mutans* to *S. mutans* was considerably higher than the frequency observed in the original interspecies transfer, group F streptococcus to *S. mutans*.

Recently, the transfer of the β plasmid was shown to occur in intergeneric matings. E. Gibson, N. Chace, and J. P. London (personal communication) have isolated clones of *Lactobacillus* species containing the β plasmid after matings with either *S.*

Table 4. Frequency of interspecies and intraspecies transfer of the β plasmid

Donor/recipient	Colony-forming units/ml after mixed incubation: Donor	Recipient	Trans-conjugant	Frequency
DR1501 (β)/DR0001	5.8×10^7	5.3×10^8	1.5×10^2	2.6×10^{-6}
DR0001 (β)/DR0001/1	1.1×10^8	2.9×10^8	1.5×10^4	1.4×10^{-4}

faecalis strain DS5 or the group F strain, DR1501 (β). The transfer frequencies observed were comparable to those reported above for intrageneric transfer.

The ease with which the β plasmid can be transferred to members of diverse species suggests its possible use as a genetic tool.

We express our appreciation to Dr. Vickers Hershfield for informing us of the transmissibility of the β plasmid and for discussing her results with the group D and group B streptococci prior to publication.

1. Falkow, S. (1975) *Infectious Multiple Drug Resistance* (Pion Limited, London), pp. 76–92.
2. Raycroft, R. E. & Zimmerman, L. N. (1964) *J. Bacteriol.* **87,** 799–801.
3. Jacob, A. E. & Hobbs, S. J. (1974) *J. Bacteriol.* **117,** 360–372.
4. Jacob, A. E., Douglas, G. J. & Hobbs, S. J. (1975) *J. Bacteriol.* **121,** 863–872.
5. Dunny, G. M. & Clewell, D. B. (1975) *J. Bacteriol.* **124,** 784–790.
6. Van Embden, J. D. A., Engel, H. W. B. & VanKlingeren, B. (1977) *Antimicrob. Ag. Chemother.* **11,** 925–932.
7. Franke, A. E., Dunny, G. M., Brown, B. L., An, F., Oliver, D. R., Damle, S. P. & Clewell, D. B. (1978) in *Microbiology—1978,* ed. Schlessinger, D. (American Society for Microbiology, Washington, DC), pp. 45–47.
8. Oliver, D. R., Brown, B. L. & Clewell, D. B. (1977) *J. Bacteriol.* **130,** 948–950.
9. Clewell, D. B., Yagi, Y., Dunny, G. M. & Schultz, S. K. (1974) *J. Bacteriol.* **117,** 283–289.
10. Wittenberger, C. L., Beaman, A. J. & Lee, L. N. (1978) *J. Bacteriol.* **133,** 231–239.
11. LeBlanc, D. J. & Hassell, F. P. (1976) *J. Bacteriol.* **128,** 347–355.
12. Currier, T. C. & Nester, E. W. (1976) *Anal. Biochem.* **76,** 431–441.
13. Meyers, J. A., Sanchez, D., Elwell, L. P. & Falkow, S. (1976) *J. Bacteriol.* **127,** 1529–1537.
14. Aaij, C. & Borst, P. (1972) *Biochim. Biophys. Acta* **269,** 197–200.
15. LeBlanc, D. J., Cohen, L. & Jensen, L. (1978) *J. Gen. Microbiol.* **106,** 49–54.