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Natural Transfer of Conjugative Transposon Tn*916* between Gram-Positive and Gram-Negative Bacteria

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The conjugative streptococcal transposon Tn*916* was found to transfer naturally between a variety of gram-positive and gram-negative eubacteria. *Enterococcus faecalis* hosting the transposon could serve as a donor for *Alcaligenes eutrophus*, *Citrobacter freundii*, and *Escherichia coli* at frequencies of 10^{-6} to 10^{-8}. No transfer was observed with several phototrophic species. Mating of an *E. coli* strain carrying Tn*916* yielded transconjugants with *Bacillus subtilis*, *Clostridium acetobutylicum*, *Enterococcus faecalis*, and *Streptococcus lactis* subsp. *diacetylactis* at frequencies of 10^{-4} to 10^{-6}. *Acetobacterium woodii* was the only gram-positive organism tested that did not accept the transposon from a gram-negative donor. The results prove the ability of conjugative transposable elements such as Tn*916* for natural cross-species gene transfer, thus potentially contributing to bacterial evolution.

It had long been assumed that no natural DNA transfer could take place between gram-positive and gram-negative bacteria. However, antibiotic resistance determinants from gram-positive bacteria are often expressed in gram-negative microorganisms, whereas the reverse is uncommon (7, 20, 40). From analysis of the nucleotide sequences and the deduced amino acid sequences of aminoglycoside and macrolide resistance genes, it has been concluded that a horizontal transfer of genetic material from gram-positive to gram-negative bacteria must have occurred (4, 39, 40, 42). These authors favored a transformationlike process.

Artificially constructed shuttle vectors were recently shown to mediate a conjugal transfer from *Escherichia coli* to a variety of gram-positive genera (25, 31, 41). These plasmids had been constructed by combining origins of replication from pBR322, pAMβ1, pIJ101, and pACYC184 with the origin of transfer of the IncP plasmids RK2 and RP4. Natural transmission of genetic information between very distantly related taxonomic groups via conjugation has been shown from *Agrobacterium tumefaciens* to plants (5, 22, 34) and from *E. coli* to yeasts (17). Strong indications for a similar process from fish to *Photobacterium leiognathi* have also been obtained (1). Prerequisites for successful exchange of genes are (i) transfer across the genus barrier and (ii) stable replication in the new host. Since in most cases plasmids from gram-positive bacteria cannot be stably maintained in gram-negative organisms, and vice versa, the ideal candidate would be a conjugative transposable element able to enter the host and integrate into its chromosome. Such a class of transposons has been discovered in streptococci and was recently extensively reviewed (12). Its most prominent representative is Tn*916*. In this report, we provide evidence for transfer of Tn*916* between gram-positive and gram-negative bacteria under natural conditions.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains, their plasmid contents, and the media used for their cultivation are shown in Table 1. When present, antibiotics were used at the following concentrations: ampicillin, 25 μg/ml; colistin methanesulfonate, 10 μg/ml; penicillin, 10 μg/ml; and tetracycline, 10 to 20 μg/ml. Media for *Clostridium acetobutylicum* and *Acetobacterium woodii* were prepared under strictly anaerobic conditions (13). Minimal medium for *A. woodii* contained, per liter, $NaHCO_3$, 1 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; KH_2PO_4, 1.76 g; $K_2HPO_4 \cdot 3H_2O$, 8.44 g; vitamin solution (10; medium 141), 2 ml; mineral salts solution (46; modified by addition of 3 mg of $NiCl_2 \cdot 6H_2O$/liter), 20 ml; $Na_2SeO_3 \cdot 5H_2O$, 3 μg; $Na_2WO_4 \cdot 2H_2O$, 4 μg; cysteine hydrochloride, 300 mg; $Na_2S \cdot 9H_2O$, 300 mg; resazurin, 1 mg; and sodium lactate, 4.5 g (pH 7.3). Defined synthetic medium for phototrophic bacteria contained, per liter, KH_2PO_4, 1 g; NH_4Cl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; NaCl, 0.4 g; $CaCl_2 \cdot 2H_2O$, 50 mg; trace element solution SL 9 (43), 1 ml; vitamin solution (46), 1 ml; and DL-malic acid, 2 g (pH 6.8). Solid media contained 1.5% (wt/vol) agar. Antibiotic medium no. 3, Elliker broth, and nutrient broth were obtained from Difco Laboratories (Detroit, Mich.); colistin methanesulfonate was from Sigma Chemie GmbH (Deisenhofen, Federal Republic of Germany).

Mating procedure. The mating procedure was developed to allow convenient handling of all strains examined. Overnight cultures were transferred into fresh medium and grown under the appropriate growth conditions to optical densities at 600 nm of 1.2 (donor) or 1.2 to 1.5 (recipient). Aliquots (1 ml) were centrifuged for 5 min in Eppendorf tubes and washed once with 1 ml of 50 mM potassium phosphate buffer (pH 7.5). The pellets were suspended in 50 μl of medium, mixed, and spotted onto sterile filter disks (Schleicher & Schüll, Dassel, Federal Republic of Germany). After 24 h of incubation at 37°C (or 30°C for *A. woodii*), the filters were transferred into 2 to 3 ml of the recipient. The number of donors and recipients was determined by serial dilution and plating of samples on media indicated in Table 1. For *A. woodii* and *C. acetobutylicum*, washing was performed with anaerobic medium (twice) and incubation was under strictly

TABLE 1. Bacterial strains and cultivation conditions

Strain	Phenotypic marker[a]	Plasmid content	Medium[b]	Source and reference
Acetobacterium woodii			MM	DSM 1030 (10)
Alcaligenes eutrophus H16		pHG1	NB	DSM 428 (10)
Bacillus subtilis			ABM 3	DSM 402 (10)
Citrobacter freundii			LB	DSM 30040 (10)
Clostridium acetobutylicum			CBM	DSM 792 (10)
Enterococcus faecalis				
OG1RF	Tc^r	pAM211	EB	D. B. Clewell (15)
			ABM 3 (solid medium)	
OG1SSp	Spc^r Str^r Tc^r	pCF10	EB	J. C. Adsit and G. M. Dunny (9)
			ABM 3 (solid medium)	
JH2-2	Cm^r Em^r Fus^r Rif^r	pIP501	EB	D. B. Clewell (15, 18)
			ABM 3 (solid medium)	
Escherichia coli				
CG120	Tc^r Ap^r	pAM120	LB	D. B. Clewell (15)
HB101			LB	DSM 1607 (3, 10)
Rhodobacter capsulatus 6950			MMM	DSM 152 (10)
Rhodobacter sphaeroides Y			MMM	DSM 160 (10)
Rhodospirillum rubrum S 1			MMM	DSM 467 (10)
Streptococcus lactis subsp. *diacetylactis* Bu2-60			EB	H. Neve (27)
			ABM 3 (solid medium)	

[a] Abbreviations: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Fus^r, fusidic acid resistance; Rif^r, rifampin resistance; Spc^r, spectinomycin resistance; Str^r, streptomycin resistance; Tc^r, tetracycline resistance. Three-letter designations indicate chromosomal resistance genes; two-letter designations represent genes carried on plasmids.

[b] Abbreviations: MM, minimal medium for *A. woodii* (see Materials and Methods); NB, nutrient broth; ABM 3, antibiotic medium no. 3; LB, Luria-Bertani medium (24); CBM, clostridial basal medium (28); EB, Elliker broth supplemented with 1.9% (wt/vol) β-glycerophosphate; MMM, minimal medium for phototrophs with malate as a carbon source (see Materials and Methods). All strains were grown at 37°C except for *A. woodii* which was cultivated at 30°C.

anaerobic conditions. Counterselection was achieved by use of penicillin (*Enterococcus faecalis* as a donor), colistin (*E. coli* as a donor), or selective malate medium (for phototrophs; *Enterococcus faecalis* as a donor).

Preparation of DNA. Chromosomal DNA was isolated as described recently (2). Plasmid pAM120 from *E. coli* CG120 was prepared by alkaline lysis as described by Maniatis et al. (24).

Restriction analysis and Southern hybridization. DNA digests with restriction enzymes were performed as recommended by the manufacturer (GIBCO/BRL GmbH, Eggenstein, Federal Republic of Germany). After electrophoresis, DNA was visualized with shortwave UV light and photographed. Fragment sizes were determined by comparing mobilities with those of a standard reference (*Pst*I-digested lambda DNA). Plasmid pAM120 containing transposon Tn*916* was labeled with biotin-11-dUTP in a nick translation reaction as recommended by the supplier (GIBCO/BRL). A second probe used in hybridization experiments with gram-negative acceptor strains consisted only of the *Hin*cII B fragment of pAM120 (8), which contains the *tetM* gene, and was labeled with biotin-7-dATP (GIBCO/BRL). Transfer of DNA from agarose gels to nitrocellulose membranes (Gelman Sciences Inc., Ann Arbor, Mich.) was performed according to Southern (33). For prehybridization and hybridization, the directions of the manufacturer were followed. Detection of labeled DNA hybrids was done according to the procedure of GIBCO/BRL, with minor modifications: (i) 0.5% (wt/vol) sodium dodecyl sulfate in the washing buffer and (ii) replacement of buffers I and II by a solution containing 0.1 M Tris hydrochloride (pH 7.5), 0.1 M NaCl, 2 mM $MgCl_2$, and 0.5% (vol/vol) Tween 20. Hybridization signals appeared within 15 to 20 min.

RESULTS

Transfer of Tn*916* from gram-positive to gram-negative bacteria. *Enterococcus faecalis* with plasmid pAM211 was chosen as a donor. This plasmid contains an insertion of Tn*916* into the naturally occurring plasmid pAD1 (15). A variety of gram-negative eubacteria that includes examples of the α, β, and γ subgroups of purple bacteria (45) served as recipients. The results of conjugation experiments are presented in Table 2. In all cases except for the phototrophs, transfer of tetracycline resistance was observed. The purity of transconjugant cultures with respect to contamination by the donor was checked (i) microscopically, (ii) by repeated streaking, and (iii) by counterselection in the presence of penicillin. A concentration of 10 μg/ml was sufficient to completely inhibit growth of *Enterococcus faecalis*, whereas all gram-negative bacteria tested were unaffected. To verify the presence of Tn*916* in the recipients, DNA was isolated

TABLE 2. Transfer of Tn*916* to gram-negative eubacteria[a]

Recipient	Transconjugants/donor[b]	Transconjugants/recipient
Alcaligenes eutrophus	1.1×10^{-6}	2.8×10^{-6}
Citrobacter freundii	4.8×10^{-6}	1.0×10^{-8}
Escherichia coli HB101	1.4×10^{-8}	6.6×10^{-7}

[a] No transfer was obtained with *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, and *Rhodospirillum rubrum*. The concentration of tetracycline used for selection was 10 μg/ml except for *Citrobacter freundii*, for which 20 μg/ml was used. Penicillin was present for counterselection of the donor at a concentration of 10 μg/ml. For all phototrophs, no penicillin but the selective malate minimal medium was used for counterselection.

[b] *Enterococcus faecalis* OG1RF(pAM211) was used as the donor. With all phototrophs, *Enterococcus faecalis* OG1SSp(pCF10) was also tried.

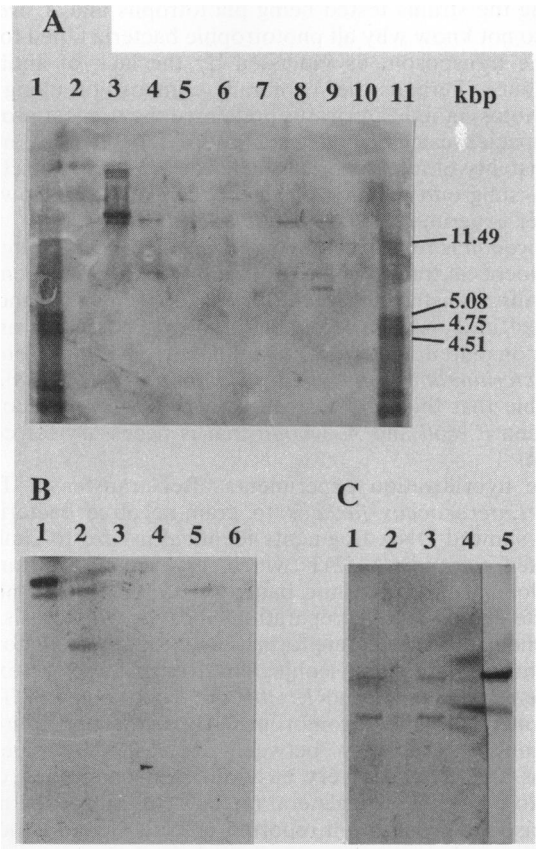

FIG. 1. Hybridization of *Hin*dIII-digested chromosomal DNA from transconjugants of several gram-negative species after mating with Tn*916*-containing *Enterococcus faecalis*. (A) Lanes: 1, biotin-labeled, *Pst*I-digested λ DNA used as a size marker; 2, no sample applied; 3, *Hin*dIII-digested pAM120; 4, *Hin*dIII-digested pAM211; 5, no sample applied; 6, *Citrobacter freundii* transconjugant; 7, *C. freundii* parent; 8 and 9, *Alcaligenes eutrophus* transconjugants; 10, *A. eutrophus* parent; 11, biotin-labeled, *Pst*I-digested λ DNA used as a size marker. Bands from the *C. freundii* transconjugant were hardly visible. Therefore, more DNA of the same transconjugant was used for hybridization as shown in panel B. (B) Lanes: 1, *Hin*dIII-digested pAM120; 2, *Hin*dIII-digested pAM211; 3, no sample applied; 4, *C. freundii* parent; 5, *C. freundii* transconjugant; 6, *C. freundii* parent. (C) Lanes: 1, *E. coli* transconjugant; 2, *E. coli* parent; 3, *E. coli* transconjugant; 4, *Hin*dIII-digested pAM211; 5, *Hin*dIII-digested pAM120.

from parent strains and two randomly picked transconjugants in each case. After digestion with *Hin*dIII, the DNAs were run on an agarose gel, blotted onto nitrocellulose membranes, and probed with biotin-labeled *tetM* gene of Tn*916*. The results are shown in Fig. 1. Only the transconjugants gave positive hybridization signals. All of them showed two bands that ran at the same position as did *Hin*dIII-digested pAM211. On agarose gels, however, plasmid bands corresponding to this plasmid were not detected. In the case of one *Alcaligenes eutrophus* transconjugant, two additional bands were visible, as expected for transposition of another copy of Tn*916* (which contains an internal *Hin*dIII site) to the chromosome.

No transfer of Tn*916* to phototrophic species was found, as judged from the lack of tetracycline-resistant colonies after mating. Additional matings were performed with an *Enterococcus faecalis* strain carrying plasmid pCF10, which contains another conjugal transposon, Tn*925*. Again, no tetracycline-resistant transconjugants were found.

TABLE 3. Transfer of Tn*916* to gram-positive eubacteria[a]

Recipient	Transconjugants/ donor[b]	Transconjugants/ recipient
Bacillus subtilis	5.5×10^{-6}	6.8×10^{-6}
Clostridium acetobutylicum	1.4×10^{-5}	5.3×10^{-5}
Enterococcus faecalis JH2-2	4.6×10^{-4}	1.1×10^{-5}
Streptococcus lactis subsp. *diacetylactis*	1.6×10^{-5}	8.3×10^{-6}

[a] No transfer was obtained with *A. woodii*. The concentration of tetracycline used for selection was 10 μg/ml except for *B. subtilis*, for which 15 μg/ml was used. Colistin methanesulfonate was present for counterselection of the donor at a concentration of 10 μg/ml.
[b] *E. coli* CG120(pAM120) was used as the donor.

Transfer of Tn*916* from gram-negative to gram-positive bacteria. Since no naturally occurring plasmid with a Tn*916* insertion is known for gram-negative bacteria, *E. coli* CG120(pAM120) was chosen as a donor. pAM120 is a pBR322-derived vector with a cloned intact element of Tn*916* (15). Since the pBR322 origin of replication is not functional in gram-positive bacteria (44), our screening only allowed selection of chromosomal insertions of the conjugative transposon in the new hosts. A variety of gram-positive eubacteria, including aerobic and anaerobic species, were chosen as recipients. They all belong to the low-G+C subdivision and represent a phylogenetically deep cluster, in contrast to the high-G+C subdivision (45). The results of the conjugation experiments are presented in Table 3. Transfer of tetracycline resistance was observed with all species tested except *A. woodii*. The purity of transconjugant cultures with respect to contamination by the donor was checked as described above except that counterselection was performed by using colistin. To obtain further evidence for Tn*916* transposition, hybridizations of chromosomal DNA preparations were done as described above. Again, only the transconjugants yielded positive signals, whereas all parent strains remained silent (Fig. 2 and 3). In all cases, more than two bands were visible, which indicated transpo-

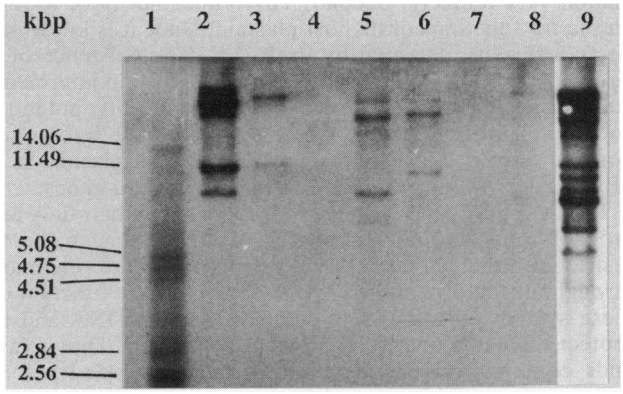

FIG. 2. Hybridization of *Hin*dIII-digested chromosomal DNA from transconjugants of several gram-positive species after mating with Tn*916*-containing *E. coli*. Lanes: 1, biotin-labeled, *Pst*I-digested λ DNA used as a size marker; 2 and 3, *Enterococcus faecalis* transconjugants; 4, *Enterococcus faecalis* parent; 5 and 6, *Bacillus subtilis* transconjugants; 7, *B. subtilis* parent; 8, *S. lactis* parent; 9, *S. lactis* transconjugant.

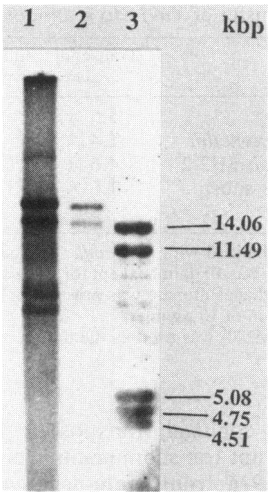

FIG. 3. Hybridization of *Hin*dIII-digested chromosomal DNA of *C. acetobutylicum* after mating with Tn*916*-containing *E. coli*. Lanes: 1 and 2, *C. acetobutylicum* transconjugants (the parent shows no hybridization with Tn*916* [2]); 3, biotin-labeled, *Pst*I-digested λ DNA used as a size marker.

sition of several copies of Tn*916* to the chromosome. The multitude of signals observed with the *Streptococcus lactis* transconjugant probably was due to an incomplete digest, as judged from the agarose gel (not shown).

DISCUSSION

The data presented in this report provide evidence that the streptococcal conjugative transposon Tn*916* is able to cross naturally the barrier between a variety of gram-positive and gram-negative eubacteria, with subsequent expression in the new host. Although we did not make any attempt to determine the nature of the transfer process (transformation versus conjugation), there are indications that favor a conjugationlike mechanism. First, *C. acetobutylicum* has been reported to possess extremely active nucleases (6, 22). Therefore, any free DNA that might be present after some cell lysis would be degraded at once, thus making transformation very unlikely. Second, Tn*916* does not have to rely on the *tra* functions of its host plasmid, since it is known to possess fertility potential by itself (12). The existence of a circular form postulated earlier as a transposition intermediate has been recently shown (32). This fertility potential could explain the transfer of Tn*916* from pAM120 to gram-positive bacteria. The remaining part of this plasmid is a pBR322 derivative and does not contain any *tra* genes.

The quantitative data on transfer reported in this study are probably only an underestimation of DNA transfer due to restriction and modification systems that are common throughout the procaryotic world. The primary function of these systems is to degrade incoming foreign DNA and to protect the cell's own genome from restriction. Thus, plasmids containing Tn*916* might have been degraded before transposition could occur. For many species used in this study, no investigations on restriction and modification systems have been done; therefore, any possible extent of restriction on transfer of Tn*916* remains a matter of speculation.

Transfer of Tn*916* could be obtained from gram-positive to gram-negative bacteria and vice versa, the only exceptions among the strains tested being phototrophs and *A. woodii*. We do not know why all phototrophic bacteria failed to take up the transposon, as indicated by the lack of antibiotic resistance. Perhaps the membrane composition along with the photosynthetic apparatus prevented the transfer process. The species tested belong to subgroups α-1 and α-3. Whether the inability of intergeneric transfer of Tn*916* is restricted to the α subgroup of purple bacteria can only be shown by further experiments that include additional species.

A. woodii is related to *Clostridium* spp. (37). Although the streptococcal transposons Tn*916* and Tn*925* have been successfully transferred from *Enterococcus faecalis* and expressed in *A. woodii* (35), this organism possesses an uncommon murein structure, so far known to be shared only by *Eubacterium limosum* and *Clostridium barkeri* (19, 38). It is possible that this atypical cell wall prevented cell contact between *E. coli* and *A. woodii* that is necessary for conjugation.

The hybridization experiments after transfer of Tn*916* from *Enterococcus faecalis* to gram-negative bacteria always showed DNA fragments identical in size to similarly digested plasmid pAM211 (which represented the transposon donor). Since plasmid bands of pAM211 could not be detected after DNA preparation and electrophoresis, the data indicate a cointegrate formation of the whole vector and the chromosome. Transconjugants from all species except *Citrobacter freundii* and *E. coli* obtained copies of Tn*916* integrated into the chromosome. Thus, the procedure for obtaining transposition between at least the respective strains and genera is very easy and convenient and could therefore be used as a general method for transposon mutagenesis. Tn*916* has been reported to be a valuable tool for targeting and cloning genes from its hosts because it excises from cloned fragments in *E. coli* in the absence of tetracycline (15). Thus, after insertion into the gene of interest, the respective fragment could be cloned into *E. coli* by selecting for the transposon-encoded resistance. After growth in the absence of the drug, excision of the transposon would result in the restoration of the original gene. It has been shown that in about half of all such events this regeneration is exact. The other 50% contained a 3-bp change at the excision site (11).

Almost all conjugative streptococcal transposable elements described so far, namely, Tn*916*, Tn*918*, Tn*919*, Tn*920*, Tn*925*, and Tn*1545*, show extensive homology (9, 12, 14, 26). Thus, they probably arose from a common ancestor. A similar element has been detected in *Clostridium difficile* (16), suggesting that intergeneric transfer might have occurred. The results presented here demonstrate that these genetic markers are easily exchanged even between phylogenetically distant genera under natural conditions (mixing of the respective strains and appropriate incubation). It remains to be shown whether similar elements can be detected in gram-negative bacteria which would be important with respect to bacterial evolution. The *tetM* tetracycline resistance determinant, originally identified in gram-positive bacteria, has been found in several gram-negative organisms, especially the genus *Neisseria* and cell wall-free species (21, 29). Recently, a *tetM* determinant with high homology to Tn*916* has been detected in the gram-negative *Fusobacterium nucleatum* that could be transferred to *Enterococcus faecalis* and other anaerobic species (29a). This report represents another example of natural transfer of conjugal transposons from gram-negative hosts to gram-positive organisms. It has been suggested that the existence of the biological entities is maintained through the action of cross-species gene transfer (36). Support for this theory

came from analysis of sequence data of antibiotic resistance genes and insertion sequences (30, 40, 42). This work lends support to the idea that transposable elements might serve as vehicles for such a gene flux (5, 40).

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