Natural Transfer of Conjugative Transposon Tn916 between Gram-Positive and Gram-Negative Bacteria

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The conjugative streptococcal transposon Tn916 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 phototropic species. Mating of an E. coli strain carrying Tn916 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 Tn916 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 transformation-like 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 Tn916. In this report, we provide evidence for transfer of Tn916 between gram-positive and gram-negative bacteria under natural conditions.

(A preliminary account of the data presented in this report was given at the Annual Meeting of the Vereinigung für Allgemeine und Angewandte Mikrobiologie, Berlin, Federal Republic of Germany, 25 to 28 March 1990.)

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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 \( \mu \)g/ml; colistin methanesulfonate, 10 \( \mu \)g/ml; penicillin, 10 \( \mu \)g/ml; and tetracycline, 10 to 20 \( \mu \)g/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\(_4\)Cl, 1 g; MgSO\(_4\) \( \cdot \)7H\(_2\)O, 0.1 g; KH\(_2\)PO\(_4\), 1.76 g; K\(_2\)HPO\(_4\) \( \cdot \)3H\(_2\)O, 8.44 g; vitamin solution (10; medium 141), 2 ml; mineral salts solution (46; modified by addition of 3 mg of NaCl, 6H\(_2\)O/liter), 20 ml; Na\(_2\)SeO\(_3\), 5H\(_2\)O, 3 \( \mu \)g; Na\(_2\)WO\(_4\), 2H\(_2\)O, 4 \( \mu \)g; cysteine hydrochloride, 300 mg; Na\(_2\)S \( \cdot \)9H\(_2\)O, 300 mg; resazurin, 1 mg; and sodium lactate, 4.5g (pH 7.3). Defined synthetic medium for phototrophic bacteria contained, per liter, KH\(_2\)PO\(_4\), 1 g; NH\(_4\)Cl, 0.5 g; MgSO\(_4\) \( \cdot \)7H\(_2\)O, 0.4 g; NaCl, 0.4 g; CaCl\(_2\) \( \cdot \)2H\(_2\)O, 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, E:lliker 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 \( \mu \)l of medium, mixed, and spotted onto sterile filter disks (Schleicher & Schüll, Dassell, 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotypic markera</th>
<th>Plasmid content</th>
<th>Mediumb</th>
<th>Source and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetobacterium woodii</td>
<td></td>
<td>pHG1</td>
<td>MM</td>
<td>DSM 1030 (10)</td>
</tr>
<tr>
<td>Alcaligenes eutrophus H16</td>
<td></td>
<td></td>
<td>NB</td>
<td>DSM 428 (10)</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td></td>
<td></td>
<td>ABM 3</td>
<td>DSM 402 (10)</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td></td>
<td></td>
<td>LB</td>
<td>DSM 30040 (10)</td>
</tr>
<tr>
<td>Clostridium acetobutylicum</td>
<td></td>
<td></td>
<td>CBM</td>
<td>DSM 792 (10)</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OG1RF</td>
<td>Tc'</td>
<td>pAM211</td>
<td>EB</td>
<td>D. B. Clewell (15)</td>
</tr>
<tr>
<td>OG1SSp</td>
<td>Spc' Str' Tc'</td>
<td>pCF10</td>
<td>EB</td>
<td>J. C. Adsit and G. M. Dunny (9)</td>
</tr>
<tr>
<td>JH2-2</td>
<td>Cm' Em' Fus' Rif'</td>
<td>pIP501</td>
<td>EB</td>
<td>D. B. Clewell (15, 18)</td>
</tr>
<tr>
<td>Espicheria coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG120</td>
<td>Tc' Ap'</td>
<td>pAM120</td>
<td>LB</td>
<td>D. B. Clewell (15)</td>
</tr>
<tr>
<td>HB101</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rhodobacter capsulatus 6950</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Rhodobacter sphaeroides Y</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Rhodospirillum rubrum S 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus lactis subsp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diacetylactis Bu2-60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Abbreviations: Ap', ampicillin resistance; Cm', chloramphenicol resistance; Em', erythromycin resistance; Fus', fusidic acid resistance; Rif', rifampin resistance; Spc', spectinomycin resistance; Str', streptomycin resistance; Tc', 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, Etticher broth supplemented with 1.9% (wt/vol) β-glycerophosphate; MM, 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.

RESULTS

Transfer of Tn916 from gram-positive to gram-negative bacteria. Enterococcus faecalis with plasmid pAM211 was chosen as a donor. This plasmid contains an insertion of Tn916 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 Tn916 in the recipients, DNA was isolated

TABLE 2. Transfer of Tn916 to gram-negative eubacteria

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Transconjugants/ donora</th>
<th>Transconjugants/ recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcaligenes eutrophus</td>
<td>1.1 × 10⁻⁶</td>
<td>2.8 × 10⁻⁶</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>4.8 × 10⁻⁶</td>
<td>1.0 × 10⁻⁸</td>
</tr>
<tr>
<td>Escherichia coli HB101</td>
<td>1.4 × 10⁻⁸</td>
<td>6.6 × 10⁻⁷</td>
</tr>
</tbody>
</table>

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 OG1RFPAM211 was used as the donor. With all phototrophs, Enterococcus faecalis OG1SSp(pCF10) was also tried.
from parent strains and two randomly picked transconjugants in each case. After digestion with HindIII, the DNAs were run on an agarose gel, blotted onto nitrocellulose membranes, and probed with biotin-labeled tetM gene of Tn916. 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 HindIII-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 Tn916 (which contains an internal HindIII site) to the chromosome.

No transfer of Tn916 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, Tn925. Again, no tetracycline-resistant transconjugants were found.

Transfer of Tn916 from gram-negative to gram-positive bacteria. Since no naturally occurring plasmid with a Tn916 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 Tn916 (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 conjugal 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 Tn916 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-

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**TABLE 3. Transfer of Tn916 to gram-positive eubacteria**

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Transconjugants/ donor*</th>
<th>Transconjugants/ recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>$5.5 \times 10^{-6}$</td>
<td>$6.8 \times 10^{-6}$</td>
</tr>
<tr>
<td>Clostridium acetobutylicum</td>
<td>$1.4 \times 10^{-3}$</td>
<td>$5.3 \times 10^{-3}$</td>
</tr>
<tr>
<td>Enterococcus faecalis JH2-2</td>
<td>$4.6 \times 10^{-4}$</td>
<td>$1.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>Streptococcus lactis subsp. diacetylactis</td>
<td>$1.6 \times 10^{-3}$</td>
<td>$8.3 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

* 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.

* E. coli CG120 (pAM120) was used as the donor.
The hybridization experiments after transfer of Tn916 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 Tn916 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. Tn916 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, Tn916, Tn918, Tn919, Tn920, Tn925, and Tn1545, 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 Tn916 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 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 belonged to subgroups α-1 and α-3. Whether the inability of intergeneric transfer of Tn916 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 Tn916 and Tn925 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.

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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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