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Transfer of the Plasmid for Exfoliative Toxin B Synthesis in Mixed Cultures on Nitrocellulose Membranes

MARVIN ROGOLSKY,[1]* BERNARD W. BEALL,[1] AND BILL B. WILEY[2]

School of Basic Life Sciences, Medical School, University of Missouri—Kansas City, Kansas City, Missouri 64108-2792,[1] and College of Medicine, University of Utah, Salt Lake City, Utah 84132[2]

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Plasmid pRW002 carries genetic determinants for exfoliative toxin B and bacteriocin R_1 synthesis. When a donor strain carrying plasmid pRW002 was mixed with a plasmidless recipient strain on a nitrocellulose membrane in accordance with the procedure used for staphylococcal conjugation, pRW002 was passed to the recipient by mixed-culture transduction. Transfer was inhibited by citrate and serotype B phage antisera but not by DNase I. Cell-to-cell contact was not required, and transfer frequencies increased more than 10-fold in the presence of small concentrations of mitomycin C. These results are consistent with pRW002 transfer in mixed cultures by transduction and not by conjugation or transformation. Immunodiffusion and DNA analyses after agarose gel electrophoresis demonstrated that transductants were exfoliative toxin B producers and housed pRW002. Since mixed-culture transfer has been reported to occur on skin, our results suggest that mixed-culture transduction might be a mechanism for the transfer of genetic determinants for pathogenicity in vivo.

Exfoliative toxin is responsible for an overlapping spectrum of clinical manifestations called the staphylococcal scalded-skin syndrome (14). Infants are very susceptible to this staphylococcal impetigo, which is characterized by a scarlatiniform rash, bullae, and a generalized exfoliation due to intraepidermal cleavage through the stratum granulosum (14). Recent evidence has indicated that exfoliative toxin is a sphingomyelinase that is different from staphylococcal beta-toxin (23). Our research group defined two distinct serotypes of exfoliative toxin in phage group II strains of *Staphylococcus aureus* (14, 18, 22). The serotypes were designated ET-A and ET-B and had approximate molecular weights of 30,000 and 29,500, respectively, and major differences in amino acid sequences (2). The genetic determinant for ET-A was found to be chromosomal, and the genetic determinant for ET-B was located on a 27 to 29-megadalton plasmid (2, 14, 18, 20, 22) which usually also carried a gene for bacteriocin R_1 ($BacR_1$) production (10, 14, 15, 17).

Restriction endonuclease patterns of ET-B plasmids were defined by Warren (20, 21) and by O'Reilly et al. (13). DNA-DNA hybridization studies were used to determine that ET-B plasmids from six different phage group II strains shared extensive nucleotide sequence homology (20). Intra-phage group II transfer of ET-B plasmids could be demonstrated after polyethylene glycol-induced protoplast fusion (10, 15).

A prototype strain used by our research group to study exfoliative toxin was UT0002. This strain carried a 27-megadalton plasmid (pRW002) with genes for ET-B and $BacR_1$ synthesis and a determinant for $BacR_1$ resistance. It also contained a chromosomal locus for ET-A synthesis and a 2.4-megadalton cadmium resistance plasmid (pRW010). The present study was initiated to determine if pRW002 had genetic determinants for self-mobilization. The known staphylococcal conjugal plasmids differ from pRW002 in carrying genes for resistance to aminoglycosides but are similar to pRW002 in having relatively large molecular weights (1, 3, 5, 11). To be certain that a plasmid is transferred by conjugation and not by transduction after donor and recipient strains are mixed and mated on a filter membrane, mixed-culture transfer must be ruled out. There is general agreement that the mechanism for mixed-culture transfer is spontaneous or general transduction (4, 9, 12). In classical transduction, transfer occurs after the recipient strain is mixed with a donor-free transducing lysate, but transduction in mixed culture occurs after populations of donor and recipient strains are directly mixed (4, 9, 12). Mixed-culture transfer and transduction but not conjugation are dependent on Ca^{2+} and are inhibited by chelators such as citrate (12). Meijers et al. (12) observed that mixed-culture transfer was dependent upon a lysogenic donor strain that carried a serological group B transducing phage and a transducible plasmid. The recipient strain had to be deficient in host-controlled restriction and could not contain an incompatible plasmid that would interfere with the maintenance of the donor plasmid.

Since we were initially attempting to demonstrate conjugal transfer of the ET-B plasmid, a unique approach was used for the mixed-culture transfer procedure. Instead of allowing transfer to proceed in mixed broth cultures (4, 8, 9, 12), we mixed donor and recipient cells on filter membranes by following a modified Forbes-Schaberg procedure (3), which is routinely used for conjugation. Donor and recipient strains were grown for 9 to 12 h on heart infusion agar (HIA) (Difco Laboratories) and then individually suspended in 50 ml of heart infusion broth (Difco) to an optical density of 150 Klett units (red filter), as determined by a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.). Twenty milliliters of each strain was mixed and passed through a 47-mm nitrocellulose (filter 0.45 μm; Millipore Corp., Bedford, Mass.). The filter then was placed, cell side down, onto the surface of an HIA plate. When mixed cultures were treated with DNase, both heart infusion broth and HIA were supplemented with 25 μg of DNase I (Sigma Chemical Co.) per ml, 10 mM $MgSO_4$, and 20 mM Tris (pH 7.5). Selected agents were sometimes added to the

* Corresponding author.

TABLE 1. *S. aureus* strains

Strain	Phenotype[a]	Phage group	Comments	Source or reference
UT0002	ET-A$^+$ ET-B$^+$ BacR_1^r Cad^r	II	Contains plasmids pRW002[b] and pRW010, which carries a gene for cadmium resistance	16
UT0017		II	Plasmidless propagating strain for phage 3B	16
BR0090	Rif^r Nov^r	II	Strain of UT0017 made resistant to rifampin and novobiocin	This work
BR0091	Ery^r	II	Strain of UT0017 made resistant to erythromycin	This work
80CR3	Cad^r	I	Restriction enzyme deficient; contains a cadmium resistance plasmid	19
BR0080	Rif^r Str^r	I	Plasmid-cured substrain of 80CR3 made resistant to rifampin and streptomycin	This work
BR0092	Nov^r	III	Strain of RN450 (11) made resistant to novobiocin	This work
BR0093	Ery^r Nov^r Cad^r	I	Strain of 80CR3 made resistant to erythromycin and novobiocin	This work
BR0017	Rif^r Nov^r ET-B$^+$ BacR_1^r	II	Strain of BR0090 which received pRW002 from strain UT0002 in mixed cultures	This work
BR0081	Rif^r Str^r ET-B$^+$ BacR_1^r	I	Strain of BR0080 which received pRW002 from strain BR0017 in mixed cultures	This work
BR0082	Ery^r Nov^r ET-B$^+$ BacR_1^r	I	Strain of BR0093 which received pRW002 from strain UT0002 in mixed cultures	This work

[a] Abbreviations: ET-A$^+$, ET-A producer; ET-B$^+$, ET-B producer; BacR_1^r, resistance to BacR_1; Cad^r, resistance to cadmium; Rif^r, resistance to rifampin; Nov^r, resistance to novobiocin; Ery^r, resistance to erythromycin; and Str^r, resistance to streptomycin.
[b] Plasmid pRW002 carries a genetic determinant for ET-B and BacR_1 production and for resistance to BacR_1.

system to determine their effect on transfer. In such cases, HIA was supplemented with sodium citrate (final concentration, 0.02 M), mitomycin C (final concentration, 0.05 μg/ml), $CaCl_2$ (final concentration, 4×10^{-3} M), or 0.3 to 0.6 ml of serotype B phage antisera prior to placement of the filter with the mixed cultures.

After incubation of mixed cultures and donor and recipient controls on HIA overnight at 37°C, the filters were mixed with 7 ml of 0.85% saline to suspend the cells. These cells were diluted and plated onto selective media. Donor and recipient controls always were plated separately. Selective media contained 3 to 5% (vol/vol) crude BacR_1 to select for pRW002 transfer and one to two antibiotics to select for the recipient. BacR_1 was isolated by the procedure of Rogolsky and Wiley (17). The antibiotics used in selective media were rifampin (100 μg/ml), novobiocin (2 to 4 μg/ml), erythromycin (2 to 7 μg/ml), and streptomycin (100 μg/ml).

Plasmid DNAs from donors, recipients, and mixed-culture transductants were identified after agarose gel electrophoresis of in-well lysates by the procedure of Masterson et al. (10). Assays for ET-B and BacR_1 production (10), immunodiffusion (10), and isolation of antisera to serological group B transducing phages (6) have been previously described.

All strains used in this study are listed in Table 1. Table 2 shows that the transfer frequencies of pRW002 in mixed *S. aureus* cultures ranged from 3×10^{-8} to 5.1×10^{-7}. These frequencies were not decreased in the presence of DNase I, indicating that transfer was not mediated by transformation. It was assumed that HIA contained optimal concentrations of Ca^{2+}, since the addition of more Ca^{2+} to this agar did not increase transfer frequencies. The addition of citrate to mixed cultures completely inhibited the transfer of pRW002 (Table 2), indicating that transfer was not mediated by conjugation. The concentration of citrate used did not affect cell growth. It was also observed that the addition of antisera prepared against a serotype B transducing phage could either partially or completely inhibit the transfer of pRW002 in mixed cultures. Plasmid pRW002 was easily transferred from phage group II donors to phage group II recipients. Transfer of pRW002 from phage group II donors to phage group III BR0092 recipients was not observed. Strain BR0092 is a substrain of strain RN450, which is the prototype phage group III recipient for gene transfer in *S. aureus*. Transfer of pRW002 from phage group II donors to phage group I restriction enzyme-deficient BR0080 and BR0093 recipients was accomplished (Table 2). Mixed-culture transductants of BR0080 (BR0081) and BR0093 (BR0082) were able to pass pRW002 to other restriction enzyme-deficient phage group I strains but not to either the phage group III BR0092 strain or the phage group II BR0091 strain (Table 2).

Meijers et al. (12) observed that low levels of mitomycin C produced 10-fold increases in transfer frequencies during mixed-culture transfer. Similar results were observed in our work (Table 3). Transfer frequencies were increased by at least 10-fold. These results further indicated that mixed-culture transfer was mediated by transduction. All donors listed in Table 2 were shown to produce PFU after induction with mitomycin C. To further substantiate that transduction was the mode of transfer, it was shown that cell-to-cell contact was not required during mixed-culture transfer. Overnight cultures of BR0081 and BR0093 were filtered separately through 0.45-μm nitrocellulose filters. One filter

TABLE 2. Transfer frequencies of plasmid pRW002 in mixed cultures

Donor (phage group)[a]	Recipient (phage group)	Selective recipient marker(s)[b]	Transfer frequency[c]
UT0002 (II)	BR0090 (II)	Rif^r Nov^r	1.1×10^{-7}[d]
UT0002 (II)	BR0091 (II)	Ery^r	2.5×10^{-7}
UT0002 (II)	BR0080 (I)	Rif^r Str^r	3×10^{-8}
UT0002 (II)	BR0092 (III)	Nov^r	0
BR0017 (II)	BR0091 (II)	Ery^r	5.1×10^{-7}[d]
BR0017 (II)	BR0080 (I)	Rif^r Str^r	3.4×10^{-8}
BR0017 (II)	BR0093 (I)	Ery^r Nov^r	1×10^{-7}
BR0081 (I)	BR0093 (I)	Ery^r Nov^r	2.6×10^{-7}[d]
BR0081 (I)	BR0092 (III)	Nov^r	0
BR0082 (I)	BR0080 (I)	Rif^r Str^r	6×10^{-8}
BR0081 (I)	BR0091 (II)	Ery^r	0

[a] The selective donor marker was always resistance to BacR_1. All donor strains were shown to be lysogenic by their ability to produce PFU after induction with mitomycin C.
[b] See Table 1, footnote *a*, for abbreviations.
[c] Expressed as the number of mixed-culture transductants per recipient cell.
[d] No transfers were observed in the presence of citrate.

was placed cell side down on HIA, and the second, smaller-sized filter was placed cell side down on the first filter. Under these conditions, pRW002 was transferred at a frequency of 10^{-7}.

Plasmid DNAs from representative donors, recipients, and mixed-culture transductants are shown in Fig. 1. All $BacR_1$-resistant mixed-culture transductants tested (14, 18) from the crosses listed in Table 2 were shown to carry pRW002. The cadmium resistance plasmid pRW010 in strain UT0002 donors was not cotransferred. Mixed-culture transductants from the crosses listed in Table 2 were tested for $BacR_1$ and ET-B production. All of these strains were strong $BacR_1$ producers, and 92% were shown to be ET-B producers. Immunodiffusion analyses (Fig. 2) revealed that two mixed-culture BR0091 transductants (BR0500 and BR0501) which received pRW002 from strain UT0002 both produced a toxin which was serologically identical to a purified preparation of ET-B. Recipient strain BR0091 produced no ET-B.

We have demonstrated in these studies that mixed-culture transfer of plasmids by transduction can indeed occur during the procedure which is routinely used for staphylococcal conjugation. It is therefore important to consider mixed-culture transfer in all conjugation studies. Our results indicate that pRW002 was transferred by transduction in mixed cultures and not conjugation because transfer (i) was inhibited by citrate; (ii) was increased more than 10-fold in the presence of mitomycin C; (iii) did not require cell-to-cell contact; and (iv) was inhibited by serotype B phage antisera. In previous studies, all plasmids exchanged by mixed-culture transfer were observed to carry genetic determinants for antibiotic resistance and to have low molecular weights (4, 8, 9, 11, 12). However, pRW002 has a large mass (27 megadaltons) and no known genetic determinants for antibiotic resistance.

Mixed-culture transfer can be considered to be very clinically significant because it has been reported to occur in vivo on the skin (7, 8, 11). This indicates that normal flora staphylococci can serve as reservoirs for antibiotic resistance plasmids that can be passed to pathogens by mixed-culture transfer as well as by conjugation. Our results now further indicate that mixed-culture transfer might provide a mechanism for the in vivo exchange of genetic determinants for pathogenicity.

TABLE 3. Effect of low levels of mitomycin C on the transfer of plasmid pRW002 in mixed cultures

Donor[a]	Recipient	Selective recipient marker(s)[b]	Transfer frequency[c]	
			With mitomycin C[d]	Without mitomycin C
UT0002	BR0090	Rif^r Nov^r	9×10^{-7}	7.3×10^{-8}
UT0002	BR0090	Rif^r Nov^r	1.5×10^{-6}	2.5×10^{-8}
UT0002	BR0080	Ery^r	3×10^{-6}	3.5×10^{-7}

[a] The selective donor marker was always resistance to $BacR_1$.
[b] See Table 1, footnote *a*, for abbreviations.
[c] Expressed as the number of mixed-culture transductants per recipient cell.
[d] Mitomycin C was incorporated into HIA to a final concentration of 0.05 μg/ml.

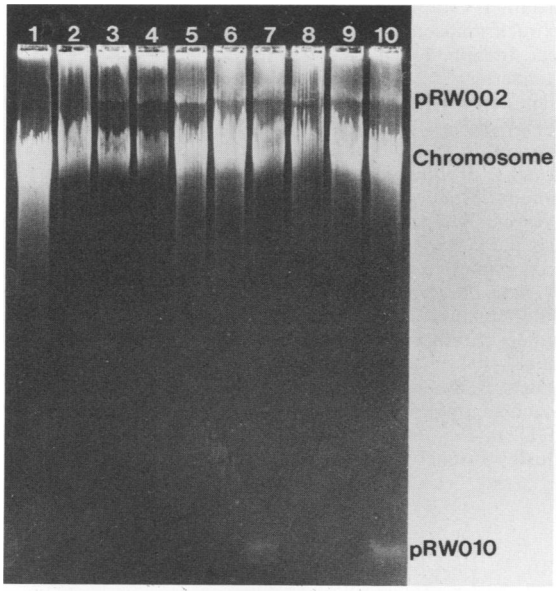

FIG. 1. Agarose gel electrophoresis of DNAs from donor, recipient, and transductant strains used for the transfer of pRW002 in mixed cultures. Lanes: 1, DNA from recipient strain BR0091 (non-ET-B producing [$ET\text{-}B^-$], non-$BacR_1$ producing [$BacR_1^-$]); 2, DNA from recipient strain BR0080 ($ET\text{-}B^-$, $BacR_1^-$); 3, DNA from pRW002-carrying donor transductant strain BR0017 (ET-B producing [$ET\text{-}B^+$], $BacR_1$ producing [$BacR_1^+$]), which resulted from the transfer of pRW002 from strain UT0002 to strain BR0090; 4, DNA from pRW002-carrying donor transductant strain BR0081 ($ET\text{-}B^+$, $BacR_1^+$), which resulted from the transfer of pRW002 from strain BR0017 to strain BR0080; 5 and 8, DNA from pRW002-carrying donor transductant strain BR0082 ($ET\text{-}B^+$, $BacR_1^+$), which resulted from the transfer of pRW002 from strain UT0002 to strain BR0093; 6 and 9, DNA from two $ET\text{-}B^+$, $BacR_1^+$ mixed-culture transductants of strain BR0091, which received pRW002 from strain BR0017; 7 and 10, DNA from pRW002-carrying donor strain UT0002 ($ET\text{-}B^+$, $BacR_1^+$).

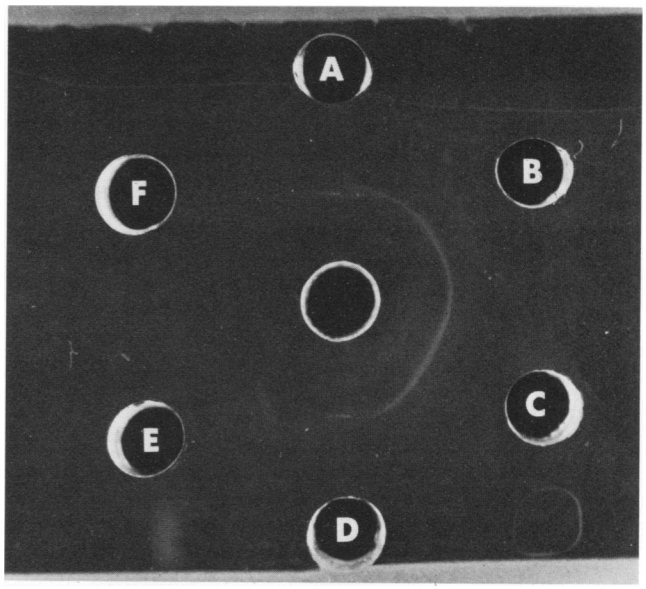

FIG. 2. Immunodiffusion analysis of ET-B produced by ET-B-producing mixed-culture transductants which resulted from the transfer of pRW002 from strain UT0002 to strain BR0091. The center well contains anti-ET-B serum. Peripheral wells B and C contain extracts of ET-B produced by ET-B-producing mixed-culture transductant strains BR0500 and BR0501. Peripheral wells A and D contain purified preparations of ET-B. Well E contains saline, and well F contains an extract of recipient strain BR0091.

Transfer of ET-B plasmids by either protoplast fusion (10, 15) or mixed-culture transfer (Table 2) appears to be limited to other phage group II and restriction enzyme-deficient phage group I recipients. It is possible that the restriction-modification systems of phage group II staphylococci are very different from those of other staphylococci (19). This might have been one of the factors which prevented the establishment of pRW002 in other recipients during intergroup transfers. We have frequently observed that plasmids from phage group II strains have much difficulty in maintaining themselves in non-phage group II environments.

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