Staphylococcal plasmids that replicate and express erythromycin resistance in both *Streptococcus pneumoniae* and *Escherichia coli* 
(heterospecific gene exchange/antibiotic resistance/ transformation/insertion sequences)

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**ABSTRACT** Plasmid pSA5700 from *Staphylococcus aureus* coding for erythromycin (Em⁺) and chloramphenicol (Cm⁺) resistance was transformed into *Streptococcus pneumoniae*. High-copy-number and Em⁺ constitutive mutants of this plasmid were isolated. Transformation frequencies in *S. pneumoniae* as high as 70% were obtained with a constitutive plasmid as donor DNA, into a recipient cell containing a resident, inductive, high-copy-number plasmid. With the aid of these high frequencies, the site of constitutive mutations could be mapped via a simple marker rescue technique that uses purified restriction endonuclease-generated fragments. One of the Em⁺ constitutive mutants, pFB9, a plasmid originating from a Gram-positive host, was shown to replicate and express Em⁺ and Cm⁺ in a Gram-negative organism, *E. coli*. Four derivatives of pFB9 containing large (0.6–0.9 megadalton) insertion sequences that arose spontaneously in *E. coli* demonstrated unusual transforming activity, as well as enhanced Em⁺, in *E. coli*. The inserted elements mapped to the region of the Em⁺ gene. Three of these inserted elements had the size and restriction patterns of insertion sequence IS1, IS2, and IS5. Plasmid pFB9 and derivatives are useful for isolation of new insertion sequences and for comparison of gene expression and illegitimate recombination between Gram-positive and Gram-negative species.

Bacteria have evolved complex and intricate mechanisms for the exchange of genetic material, not only among members of their own species but also among distantly related families (1–3). In general, plasmids isolated from Gram-positive host species [hereafter referred to as Gram(+) plasmids] have not been seen to replicate autonomously in a Gram-negative (Gram(−)) host nor vice versa.

We describe a Gram(+) plasmid, pFB9, that can replicate and express erythromycin resistance (Em⁺) in *Escherichia coli* (Gram(−)). The plasmid-encoded Em⁺ gene product, which N⁺-dimethylates a specific adenine in 23S ribosomal RNA (4, 5), is constitutively expressed in *Streptococcus pneumoniae* but only weakly expressed in *E. coli*. Insertion derivatives of pFB9 with increased Em⁺ in *E. coli* were isolated. These insertion derivatives contain *E. coli* insertion sequence (IS) elements. Illegitimate recombination with an IS element may represent a mechanism for increasing foreign gene expression in heterologous bacteria.

**MATERIALS AND METHODS**

Bacterial Strains, Plasmids, Media, and Antibiotics. *E. coli* strains K17 (C600), K19, K37, and K38 have been described (6, 7). *S. pneumoniae* strain FB1 (8) contains a low-level erythromycin resistance marker (allowing for proper induction) in a nuclease-deficient background. Plasmids pSA5700 (9) and pBR325 (10) were gifts of R. P. Novick and F. Bolivar, respectively. *E. coli* strains were grown in fortified broth (11) or tryptone broth (12). *S. pneumoniae* strains were grown in CPH8 medium (13) containing 0.8% bovine serum albumin (C + Alb) or 0.1% yeast extract (Difco) (C + Y + Alb) or both. Tylosin tartrate, Iloxicam glucoside (erythromycin), and lincomycin were generous gifts from R. Hamill of Eli Lilly and M. Guthans of Upjohn, respectively.

Plasmid DNA, Enzymes, and Electrophoresis. Plasmid DNA was prepared from *E. coli* by the method of Moses et al. (11) and from *S. pneumoniae* by a modification of published procedures (14, 15). Restriction endonuclease Sau 3A was purchased from Bethesda Research Laboratories. All other enzymes were obtained from New England BioLabs. Digestions were performed in the recommended buffers. Agarose gel electrophoresis, both analytical and preparative (with low-melting agarose), was as described (8, 16).

**Isolation of Plasmid Mutants and Transformation.** High-copy-number and constitutive plasmid mutants of pSA5700 were isolated and characterized by disk diffusion halo patterns as well as plasmid yield as described by Weisblum et al. (17). *S. pneumoniae* were grown to the competent state, activated, and transformed with plasmid DNA as described (8). When the recipient strains carried a resident plasmid, antibiotic resistance was induced in liquid culture. After addition of DNase, 2 ml of C + Y media that contained inducing erythromycin at 0.1 µg/ml was added. Cells were incubated at 37°C for 90 min. Transformants were selected by plating in either lincomycin at 1 µg/ml, tylosin at 15 µg/ml, erythromycin at 10 µg/ml, chloramphenicol at 5 µg/ml, or streptomycin at 120 µg/ml, depending on the transforming DNA.

*E. coli* transformation was by the CaCl₂ technique (18). Cells were diluted 6-fold with tryptone broth after the heat shock, and incubation was continued at 37°C for 90 min to allow for expression of antibiotic resistance markers. Transformants were selected by plating on agar containing chloramphenicol at 20 µg/ml or erythromycin at 1,000 µg/ml.

**RESULTS**

Plasmids pC194 and pE194, coding for chloramphenicol resistance (Cm⁺) and Em⁺, respectively, were isolated from *Staphylococcus aureus* (19, 20) and fused in *vivo* (9), producing plasmid pSA5700. This plasmid has been introduced into *S. pneumoniae* (8).

**Characterization of High-Copy-Number and Constitutive Mutants of Plasmid pSA5700.** Fig. 1 shows the genealogy of plasmid pSA5700 mutants isolated in *S. pneumoniae*. High-

Abbreviations: MDal, megadaltons; Em⁺, erythromycin resistance; Cm⁺, chloramphenicol resistance; Ty₁, tylosin resistance; Lin⁺, lincomycin resistance; Sm⁺, streptomycin resistance; Gram(−), Gram-positive; Gram(+), Gram-negative; IS, insertion sequence.
copy-number mutants (pFB2 and pFB3) were selected by increased Cm\(^r\). Em\(^r\) constitutive mutant plasmids pFB4 and pFB9 [formerly called pSA5700 Lc9 (8)] conferred resistance to lincomycin and tylosin (two noninducing drugs) in the absence of inducing erythromycin. Copy number and constitutive mutations were shown to be plasmid-encoded and could be distinguished as described (17).

A restriction map of pFB9 shown in Fig. 2 was constructed by using double digests as described (21, 22). Values for molecular weights agree with published values for parent plasmids pC194 and pE194 (5, 9, 21, 22).

Mapping of Constitutive Mutations by Using High Frequency Transformation. Table 1 shows transformation of the pFB9 Em\(^r\) constitutive marker (coding for Linc\(^r\) and Tyi\(^r\)) into pneumococcal strains carrying a homologous resident plasmid. Transformation frequency increased 700-fold when the recipient contained high-copy-number plasmid pFB3 compared with cells lacking the plasmid. Many restriction endonuclease-generated fragments of pFB9 were assayed by using high frequency transformation to map the Em\(^r\) constitutive marker (Fig. 3). Fragments with high transforming activity (i.e., Hha I fragment A) contain the mutation internally, whereas fragments with relatively low activity (i.e., Bcl I) have the mutation near the terminus. The constitutive mutation of pFB9 was determined to be within the overlap region of Hae III fragment B and Hpa II fragment C, probably closer to the Hae III site (based on relative frequencies) (Fig. 3). This conclusion was strengthened by Mbo

**Table 1. High frequency transformation of S. pneumoniae**

<table>
<thead>
<tr>
<th>Recipient cells*</th>
<th>DNA, 1 (\mu)g/ml</th>
<th>Frequency of transformation, %</th>
<th>Sm(^r)</th>
<th>Em(^r)</th>
<th>Tyi(^r)</th>
<th>Linc(^r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB1</td>
<td>pFB9</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FB1 (pSA5700)(^d)</td>
<td>pFB9</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>FB1 (pFB3)(^d)</td>
<td>pFB9</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>FB1 (pFB3)(^d)</td>
<td>pFB9</td>
<td>72</td>
<td>72</td>
<td>65</td>
<td>65</td>
<td></td>
</tr>
</tbody>
</table>

*Shown as strain (plasmid). Cells were activated as described. Transformation was for 30 min.

*Expression of antibiotic resistance was in liquid media and selection of transformants was as described. Viable titer was \(3 \times 10^7\) colony forming units/ml (100%).

\(^d\)R6S chromosomal DNA carries the streptomycin resistance (Sm\(^r\)) marker.

\(^d\)Low-copy-number plasmid.

\(^d\)Strains containing pSA5700 or pFB3 are already erythromycin resistant.

\(^d\)High-copy-number plasmid.
transformation into E. coli K38 (and selecting large colonies; see below) or by transformation of higher molecular weight DNA into S. pneumoniae (Fig. 4). From sixteen independent colonies examined, a total of four different plasmids was isolated that code for Em* and Cm* and breed true in both S. pneumoniae and E. coli. One isolate of each of the four plasmids was selected for further study. The four plasmids were designated pBB1 (5.0 Mdal), pBB2 (5.2 Mdal), pBB3 (5.0 Mdal), and pBB4 (5.3 Mdal) (Fig. 1).

Isolated colonies of E. coli that contained only pFB9 could be obtained from the original K37 culture by plating on erythromycin at 1,000 µg/ml, which yielded 6 large colonies (average diameter, 2 mm) to every 100 small colonies (average diameter, 0.3 mm). Plasmid DNA prepared from small colonies yielded pure pFB9, whereas larger colonies yielded pure higher molecular weight plasmids (pBB1–pBB4). Pure pFB9 from E. coli and S. pneumoniae have identical restriction patterns when the DNA is cut by Sau 3A, Hae III, Hha I, Hinfl, Alu I, HindIII, and Pst I, indicating that their structure is not necessarily altered by introduction into a new host. E. coli and S. pneumoniae DNAs differed only in sensitivity to restriction enzymes that cut the sequence C-A-T-C (Mbo I, Sau 3A) because the E. coli DNA is methylated at that site by dam methylase and S. pneumoniae DNA is not (24, 25). Despite differences in methylation, there is no restriction of plasmid DNA prepared from E. coli upon transformation into S. pneumoniae (25).

Transformation of Various E. coli Strains by pFB9. We tested pFB9 and pBB1–pBB4 for transforming ability into two isogenic E. coli strains: K17(F−) and K19(F+)(Table 2). Transformation of the male (F+) strain K19 by plasmids pFB9 and pBB1–pBB4 was 50-fold to 200-fold more efficient than the female (F−) strain K17 after 3 days’ growth on erythromycin at 1 mg/ml. Strains K37 and K38 (Hfr)—other male strains—also showed dramatically higher transformation frequencies than did F+ strains.

The virtual inability of pFB9 to transform K17 could be due to difficulties with either replication, establishment, or expression of the plasmid in that strain. To distinguish between these three factors we constructed four hybrid plasmids of pFB9 and pBR325 fused at their Pst I and HindIII sites in both orientations (26). In these hybrids, replication and establishment are pro-

**Fig. 4.** Agarose gel electrophoresis of plasmids pFB9, pBB1, and pBB2. Lane A, HindIII digest of λ phage DNA. Lanes B–D, DNA from S. pneumoniae. B, pFB9; C, pBB1; D, pBB2. Lane E, Plasmid DNA prepared from E. coli showing major band of pFB9 and two minor bands of pBB1/pBB3 and pBB2/pBB4. Electrophoresis was on 0.8% agarose.

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**Table 2.** Transformation of E. coli strains K17(F−) and K19(F+) to Em* by various pFB9 derivatives

<table>
<thead>
<tr>
<th>DNA source</th>
<th>DNA source</th>
<th>DNA</th>
<th>Transformants per µg DNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>pBR325</td>
<td>K17</td>
<td>2.6 × 10^5</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>pFB9</td>
<td>K17</td>
<td>0</td>
</tr>
<tr>
<td>E. coli</td>
<td>pFB9</td>
<td>K17</td>
<td>2.5 × 10^5</td>
</tr>
<tr>
<td>E. coli</td>
<td>pBB1</td>
<td>K17</td>
<td>2.9 × 10^5</td>
</tr>
<tr>
<td>E. coli</td>
<td>pBB2</td>
<td>K17</td>
<td>3.2 × 10^5</td>
</tr>
</tbody>
</table>

*CaCl2-treated E. coli cells (0.3 ml) were prepared and transformed as described. Antibiotic resistance markers were allowed to express (90 min at 37°C) and transformants were scored after growth for 72 hr on erythromycin at 1,000 µg/ml.

1Transformants of pBR325 were selected on plates containing chloramphenicol at 20 µg/ml.
2These transformants gave large colonies (average diameter, 4 mm) compared to other erythromycin-resistant transformants (average diameter, 0.5 mm) after growth for 72 hr on plates containing erythromycin at 1,000 µg/ml.
vided by the pBR325 replicon, and only Em$^R$ expression is contributed by the pFB9 moiety. None of the four hybrids expressed Em$^R$ in K17($F^+$), whereas all expressed appropriate pBR325 genes. In K19($F^+$) all four chimeric plasmids express Em$^R$. When we selected for Em$^R$ (as well as a pBR325 drug resistance marker), pBB3-pBR325 hybrids were found (26). Thus, Em$^R$ expression appears to be responsible for pFB9's failure to transform K17, and this can be overcome by the insertion in pFB3.

Characterization of Insertion Plasmids pBB1, pBB2, pBB3, and pBB4. Transformation of E. coli to Em$^R$ was 30- to 100-fold higher with pBB1, pBB3, or pBB4 DNA than with pFB9 DNA (Table 2). Selection of Cm$^R$ gave approximately equal frequencies of transformation for pFB9 and insertion derivatives. (These frequencies were less than 10% of those obtained on erythromycin; data not shown.)

Furthermore, K19 E. coli transformed by pBB1, pBB3, and pBB4 gave large colonies (average diameter, 4 mm), whereas those from pFB9 (and pBB2) gave small colonies (average diameter, 0.5 mm) after growth for 72 hr on erythromycin plates, suggesting that the former had greater resistance to erythromycin.

In a more direct assay of the relative strengths of Em$^R$ provided by the different plasmids, minimum inhibitory concentrations were determined. The E. coli strains used are naturally 1,000-fold more resistant to erythromycin than Gram$(+)$ species. In strain K19, plasmid pFB9 conferred resistance to tylosin at 8 mg/ml and pBB2 grew in tylosin at 64 mg/ml, whereas pBB1, pBB3, and pBB4 conferred resistance to tylosin at 125 mg/ml. [Tylosin was used due to its greater solubility; all strains grew in erythromycin-saturated (8 mg/ml) media.]

Restriction maps of the insertion plasmids were determined and are shown in Fig. 5 B–D. Digestion with Sau 3A localized the insertions within a small region 5' of the Em$^R$ marker of pFB9. Plasmids pBB1, pBB2, and pBB4 contain insertions of 0.6, 0.8, and 0.9 MDal. Their size and restriction sites correspond to insertion elements IS1, IS5, and IS2, respectively (27–29). These assignments were confirmed by single and double digests with 10–15 enzymes, including Hae III, Hpa II, Hku I, and Sau 3A. Plasmid pBB3 harbors an unidentified insertion element (0.6 MDal) that contains at least two Hae II sites.

**FIG. 5.** Restriction maps of pSA5700 insertion derivatives. (A) Restriction map of constitutive plasmid pFB9 (and pFB4) showing larger Mbo I F fragment than inducible plasmid pFB3. The constitutive mutation was mapped to the same region, depicted as M, by transformation as shown in Fig. 3. (B–D) Restriction maps of plasmids pBB1 (B), pBB2 (C), and pBB4 (D), three derivatives of plasmid pFB9 that show enhanced resistance to erythromycin and tylosin in E. coli. Plasmid pBB1 contains IS1 (0.6 MDal) inserted in the Mbo I G fragment of pFB9, pBB2 contains IS5 (0.8 MDal) inserted near the Mbo I/G/E site, and pBB4 contains IS2 (0.9 MDal) inserted within the Mbo I F fragment. Position of each restriction site indicated within the insertion sequence was confirmed by double digestion with at least three of the following enzymes: HincII, Pst I, Bgl I, Hae III, and Hha I. Over 200 restriction digests were analyzed to obtain the above maps.

**DISCUSSION**

Reanney has proposed that the exchange of extrachromosomal elements (episomes, plages, and plasmids) is an important mechanism in the evolution of microorganisms (2). The transfer of plasmids between Gram$(+)$ and Gram$(−)$ species extends the exchange of genetic information and has previously been accomplished with hybrid Gram$(+)/$Gram$(−)$ plasmids fused in vitro (30–32). Recently, Goze and Ehrlich showed that one such hybrid plasmid (a pBR322-pC194 chimera) could replicate in E. coli even though the Gram$(−)$ pBR322 replication origin had been removed (33). Paradoxically, replication of pure pC194 or other Gram$(+)$ plasmids (pT127, pUB112, pC221, pHV400) could not be demonstrated in E. coli (33).

This work characterizes the replication and expression of a Gram$(+)$ plasmid, pFB9, in two diverse bacteria, S. pneumoniae and E. coli. DNA transformation between and within these two hosts was instrumental in isolation and characterization of several insertion mutants with altered Em$^R$ expression.

When DNA of homogenous sequence—either cloned chromosomal (34) or plasmid (ref. 8 and this work)—was used as donor into an S. pneumoniae recipient containing homologous DNA, transformation frequencies as high as 50–70% were obtained. We used this high frequency transformation to biolog-
Weisblum, sequences new insertion of expression with eukaryotic insertion IS5 by insertion yet the determinant. Such one in that the frequencies, presumably originated mutations, which also appeared elements pFB9 23). All pically insert into S. -&\textsuperscript{1}DNA sequence analysis (5, 13). Promoter-like activity has been demonstrated by Ghosal et al. for IS2 in orientation II (29). Plasmid pB4 contains IS2 in this orientation, where it presumably increases transcription of the Em\textsuperscript{R} gene. By analogy, we believe that IS1 and IS5 (in pB1 and pBB2, respectively) also increase this transcription. Plasmid pBB2 displays intermediate Em\textsuperscript{R} expression; this may be due to its greater distance from the Em\textsuperscript{R} gene. Other insertion elements—IS3, Tn5, IS2-6 (36–38)—have turned on distal genes in E. coli. This has been attributed to either an internal promoter, or a rearrangement within the sequence to create a promoter, or promoter formation at the insertion sequence boundary. Our finding that at least three E. coli insertion sequences can increase Em\textsuperscript{R} expression by inserting upstream from the Em\textsuperscript{R} gene is similar to recent findings with eukaryotic transposable elements (39, 40). Ty-1, a transposable element found in yeast, can also apparently increase expression of downstream genes (40).

Plasmid pFB9 may provide a simple method for isolation of new insertion sequences (such as the unidentified insertion in pBB3) that increase gene expression. Plasmids containing insertion sequences may be easily purified by transformation of higher molecular weight bands into S. pneumoniae or by retransformation of total plasmid into E. coli where insertion plasmids exhibit a higher transformation frequency. Such insertion plasmids should further our understanding of heterologous gene expression.

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