Intraspecific and Intergeneric Mobilization of Non-conjugative Resistance Plasmids by a 24·5 Megadalton Conjugative Plasmid of *Neisseria gonorrhoeae*

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pLE2451, a 24·5 megadalton conjugative plasmid from *Neisseria gonorrhoeae*, was capable of efficiently mobilizing gonococcal β -lactamase plasmids between gonococci and from gonococci to *Haemophilus influenzae* and restriction-deficient *Escherichia coli*. Donor strains of *N. gonorrhoeae* carrying pLE2451 were also found to be capable of mobilizing a variety of non-conjugative plasmids originally derived from enteric bacteria or *Haemophilus* species when such plasmids were resident in *E. coli*. Nevertheless, pLE2451 was not detected physically in *E. coli* or *H. influenzae* transconjugants. This suggests that the plasmid is unstable in these hosts but survives transiently to provide transfer functions for mobilization. The proficiency of pLE2451 in promoting intraspecific and intergeneric mobilization was not paralleled by pUB701, pRI234 and pFR16017, a series of conjugative plasmids derived originally from *Haemophilus* species. These plasmids were incapable of mobilizing even *Haemophilus* β -lactamase plasmids, such as RSF0885, between *Haemophilus* species.

INTRODUCTION

Penicillin-resistant strains of *Neisseria gonorrhoeae* that produce the TEM β -lactamase contain non-conjugative plasmids of either 4·4 or 3·2 megadaltons (Elwell *et al.*, 1977a; Roberts *et al.*, 1977). The vast majority of gonococcal isolates, whether or not they carry β -lactamase plasmids, contain a 2·6 megadalton multicopy plasmid of unknown function (Davies & Normark, 1980; Mayer *et al.*, 1974; Roberts *et al.*, 1979). In addition, many strains of *N. gonorrhoeae*, isolated from diverse geographical locations, carry larger conjugative plasmids of about 24·5 megadaltons (Roberts *et al.*, 1979; Sox *et al.*, 1978; Tenover *et al.*, 1980). These large plasmids form a group of closely related DNA molecules sharing a common core sequence of 70 to 100% (Roberts *et al.*, 1979). They have been referred to as gonococcal sex factors and are capable of mobilizing the 4·4 megadalton β -lactamase plasmid to other gonococci, to *Neisseria flava* and to restriction-deficient *Escherichia coli* (Eisenstein *et al.*, 1977; Roberts & Falkow, 1977; Sox *et al.*, 1978).

Molecular studies have indicated that the β -lactamase plasmids of *N. gonorrhoeae* are related in base sequence to small β -lactamase plasmids isolated from *Haemophilus influenzae* (Laufs *et al.*, 1979), *Haemophilus parainfluenzae* (Roberts *et al.*, 1977) and *Haemophilus ducreyi* (Brunton *et al.*, 1979). This has prompted speculation that *N. gonorrhoeae* may have acquired its β -lactamase plasmids by genetic transfer of plasmids already existing in the *Haemophilus* gene pool (Laufs *et al.*, 1979; Roberts *et al.*, 1977; Sparling *et al.*, 1978). In an attempt to understand the routes by which gonococcal resistance plasmids may have evolved

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we have investigated the ability of the 24.5 megadalton plasmid pLE2451 (Elwell & Falkow, 1977) to mobilize a variety of non-conjugative plasmids in intraspecific and intergeneric matings.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are shown in Table 1.

Antibiotics. Ampicillin and carbenicillin were gifts of Beecham Pharmaceuticals, Brockham Park, Surrey. Tetracycline and chromogenic cephalosporin 87/312 were gifts of Glaxo Research Ltd, Greenford, Middx. All other antibiotics were obtained from Sigma.

Media and growth conditions. *Neisseria gonorrhoeae* and *Haemophilus influenzae* were routinely grown in an atmosphere of 5–10% (v/v) CO₂ at 37 °C on chocolate agar prepared from Columbia Agar Base (Oxoid) and 10% (v/v) horse blood. This medium was supplemented when required with rifampin (5 µg ml⁻¹), nalidixic acid

Table 1. *Bacterial strains and their plasmids*

Strain	Chromosomal markers*	Plasmid designation	Mol. mass (megadalton)	Resistance pattern†	Reference/source
<i>N. gonorrhoeae</i> 1L261	—	pLV11 —	3.2 2.6	Ap —	This study; Royal Liverpool Infirmary
<i>N. gonorrhoeae</i> 1L432	—	— pLV10 —	24.5 4.4 2.6	— Ap —	This study; Royal Liverpool Infirmary
<i>N. gonorrhoeae</i> 1L433	—	pLV12 —	4.4 2.6	Ap —	This study; Royal Liverpool Infirmary
<i>N. gonorrhoeae</i> KH45	—	pLE2451	24.5	—	Elwell & Falkow (1977)
<i>N. gonorrhoeae</i> 1L403	Rif ^r Nal ^r	—	2.6	—	This study
<i>H. influenzae</i> UB2806	Str ^r	—	—	—	Saunders <i>et al.</i> (1978)
<i>H. influenzae</i> UB2808	Str ^r Nal ^r	—	—	—	Nal ^r derivative of UB2806
<i>H. influenzae</i> FR16017	—	pFR16017	33	Tc	Kaulfers <i>et al.</i> (1978)
<i>H. influenzae</i> UB2811	—	pUB701	31	Tc	Elwell <i>et al.</i> (1977b)
<i>H. influenzae</i> HC234	—	pRI234	38	Cm Tc	van Klingeren <i>et al.</i> (1977)
<i>E. coli</i> KH802	<i>rpoB</i> <i>hsdR_k⁻ M_k⁺</i>	—	—	—	Wood (1966)
<i>E. coli</i> UB1005	<i>met nalA</i>	—	—	—	Grinsted <i>et al.</i> (1972)
<i>E. coli</i> J53(RSF0885)	<i>met pro nalA</i>	RSF0885	4.1	Ap	Roberts <i>et al.</i> (1977)
<i>E. coli</i> J53(pJB1)	<i>met pro nalA</i>	pJB1	6.0	Ap	Brunton <i>et al.</i> (1979)
<i>E. coli</i> 1L40	—	NTP1	5.6	Ap	Smith <i>et al.</i> (1974)
<i>E. coli</i> 1L44	<i>nalA</i>	NTP5	6.5	Tc	Smith <i>et al.</i> (1974)
<i>E. coli</i> 1L190	<i>strA</i>	NTP16	5.7	Ap Km	Anderson <i>et al.</i> (1977)
<i>E. coli</i> C600(ColE1.Tn501)	—	ColE1.Tn501	10.2	Hg	P. M. Bennett

* Resistance to: Rif^r, *rpoB*, rifampin; Nal^r, *nalA*, nalidixic acid; Str^r, *strA*, streptomycin.

† Resistance to: Ap, ampicillin; Tc, tetracycline; Cm, chloramphenicol; Km, kanamycin; Hg, mercury.

(2 µg ml⁻¹) or penicillin G (5 µg ml⁻¹) for gonococci and with streptomycin (50 µg ml⁻¹), nalidixic acid (5 µg ml⁻¹), ampicillin (10 µg ml⁻¹) or tetracycline (4 µg ml⁻¹) for *Haemophilus* strains. *Escherichia coli* strains were grown in nutrient broth or on nutrient agar at 37 °C. The medium was supplemented with rifampin (100 µg ml⁻¹), nalidixic acid (20 µg ml⁻¹), tetracycline (10 µg ml⁻¹), HgCl₂ (15 µg ml⁻¹) or ampicillin (50 µg ml⁻¹) for resistance selection. In certain triparental matings, e.g. *N. gonorrhoeae* × *E. coli*(pLV10) × *E. coli*, ampicillin (250 µg ml⁻¹) or carbenicillin (250 µg ml⁻¹) was incorporated in the medium selective for transconjugants. These high antibiotic concentrations were required to overcome the problem of background growth resulting from the β-lactamase activity of the intermediate donor.

Agarose gel electrophoresis of plasmid DNA. Cleared lysates of bacterial cultures were prepared and subjected to electrophoresis through 1% (w/v) agarose gels essentially as described by Meyers *et al.* (1976).

Transformation of E. coli. *Escherichia coli* strains were transformed with plasmid DNA as described previously (Brown *et al.*, 1979).

Conjugation. Biparental matings were performed by mixing donor and recipient strains (10⁸ colony-forming units ml⁻¹) on 0.45 µm pore-size nitrocellulose filters (Oxoid Nuflow) and incubating the filters on the surface of chocolate agar plates for 18 h at 37 °C in an atmosphere of 5–10% (v/v) CO₂. Cells were then removed from the filters by agitation in appropriate liquid medium and plated on either chocolate agar or nutrient agar suitably supplemented with antibiotics.

In triparental matings the combined number of primary donor and intermediate recipients was 10⁸ colony-forming units. In intergeneric crosses the ratio of *N. gonorrhoeae* or *H. influenzae* primary donors to *E. coli* intermediate recipients was approximately 10:1 in order to avoid overgrowth of the latter during overnight incubation. Transfer frequencies are expressed as transconjugants per colony-forming unit of initial donor at the onset of mating. In the biparental matings deoxyribonuclease I (Sigma) was added to a final concentration of 50 µg ml⁻¹ to eliminate the possibility of plasmid transfer by transformation.

RESULTS

Transfer of pLV10 and pLV11 from N. gonorrhoeae

Our results from filter mating experiments (Table 2) confirmed the findings of others (Eisenstein *et al.*, 1977; Roberts & Falkow, 1977) that conjugative transfer of β-lactamase production in *N. gonorrhoeae* is dependent on the presence of a 24.5 megadalton plasmid. When residing in the same cell this plasmid mobilized pLV10 to other gonococci, to *H. influenzae* and to restriction-deficient (*hsdR_k*⁻*M_k*⁺), but not restriction-proficient, strains of *E. coli*. In contrast, apart from some recent isolates from the Netherlands (J. van Embden, personal communication), all natural isolates of *N. gonorrhoeae* carrying the 3.2 megadalton β-lactamase plasmid (e.g. pLV11) lack the gonococcal sex factor and consequently did not transfer penicillin-resistance to other bacteria (Table 2). A triparental mating system was therefore used to establish the mobility of pLV11. *Neisseria gonorrhoeae* strain KH45, which carries only the plasmid pLE2451 (Elwell & Falkow, 1977), could mobilize pLV11 from an intermediate donor strain of *N. gonorrhoeae* (1L261) to recipient bacteria of various species. Triparental matings were also successful for demonstrating mobilization of pLV10.

Table 2. *Transfer of penicillin resistance from N. gonorrhoeae to other bacteria*

Initial donor	Intermediate donor	Recipient	Transfer frequency	Plasmids in transconjugants (megadalton)
1L261	—	<i>N. gonorrhoeae</i> 1L403	<10 ⁻⁸	2.6
1L433	—	<i>N. gonorrhoeae</i> 1L403	<10 ⁻⁸	2.6
1L432	—	<i>N. gonorrhoeae</i> 1L403	8 × 10 ⁻⁴	24.5, 4.4, 2.6 or 4.4, 2.6
1L432	—	<i>E. coli</i> KH802	1 × 10 ⁻³	4.4
1L432	—	<i>E. coli</i> UB1005	<10 ⁻⁸	—
1L432	—	<i>H. influenzae</i> UB2808	1 × 10 ⁻⁴	4.4
KH45	<i>N. gonorrhoeae</i> 1L261	<i>N. gonorrhoeae</i> 1L403	2 × 10 ⁻⁴	24.5, 3.2, 2.6 or 3.2, 2.6
KH45	<i>N. gonorrhoeae</i> 1L261	<i>E. coli</i> KH802	1 × 10 ⁻³	3.2
KH45	<i>N. gonorrhoeae</i> 1L261	<i>H. influenzae</i> UB2808	4 × 10 ⁻⁵	3.2

Table 3. *Ability of pLE2451, present in initial donor N. gonorrhoeae KH45, to mobilize β -lactamase plasmids pLV10 and pLV11 resident in E. coli or H. influenzae*

Intermediate donor	Recipient	Transfer frequency
<i>E. coli</i> KH802(pLV10)	<i>E. coli</i> UB1005	2×10^{-3}
<i>E. coli</i> KH802(pLV11)	<i>E. coli</i> UB1005	5×10^{-4}
<i>E. coli</i> KH802(pLV10)	<i>N. gonorrhoeae</i> 1L403	$< 10^{-8}$
<i>E. coli</i> UB1005(pLV10)	<i>E. coli</i> KH802	$< 10^{-8}$
<i>H. influenzae</i> UB2806(pLV10)	<i>H. influenzae</i> UB2808	$< 10^{-8}$
<i>H. influenzae</i> UB2806(pLV10)	<i>E. coli</i> KH802	$< 10^{-8}$
<i>H. influenzae</i> UB2806(pLV10)	<i>N. gonorrhoeae</i> 1L403	$< 10^{-8}$

Approximately 50% of the penicillin-resistant transconjugant clones from either bi- or triparental intraspecific gonococcal matings were found by agarose gel electrophoresis to have acquired pLE2451 in addition to the selected β -lactamase plasmid. Such clones were themselves capable of acting as donors of pLV10 or pLV11. This was in contrast to our observation of intergeneric crosses where transconjugants of *H. influenzae* and *E. coli* contained either pLV10 or pLV11 alone.

Demonstration of transfer functions of pLE2451 in E. coli

The absence of pLE2451 from transconjugants of certain species raised the question as to whether this plasmid was unable to enter these other bacterial species or whether it was so unstable as to be lost readily in the absence of direct selection pressure. A triparental system was used in order to distinguish these possibilities: it consisted of *N. gonorrhoeae* KH45 (carrying pLE2451) as primary donor, an *E. coli* (*hsdR_k⁻M_k⁺*) intermediate donor strain (KH802) carrying either pLV10 or pLV11, and a final recipient *hsdR_k⁺M_k⁺* strain of *E. coli*. The rationale of these experiments was such that entry of pLE2451 into the intermediate donor would be unimpeded by the restriction barrier and any DNA present in this donor would become modified for transfer to the final recipient. Table 3 shows that pLE2451 was able to mobilize both pLV10 and pLV11 between strains of *E. coli* so long as the restriction-deficient (KH802) and not the restriction-proficient (UB1005) strain was used as intermediate donor. No mobilization was detected if the final *E. coli* recipient was replaced by a recipient strain of *N. gonorrhoeae*. The probable reason for this became apparent when crude culture lysates of the recipient gonococcal strain 1L403 were found to cleave pLV10 and pLV11 DNA propagated in *E. coli* or *H. influenzae* into several discrete fragments (data not shown). Cleavage patterns on plasmid and phage λ DNA indicated that this strain, together with all other strains of *N. gonorrhoeae* used in this study (unpublished results), possesses the restriction endonuclease NgoII (Clanton *et al.*, 1979).

The triparental mating technique was extended to include *H. influenzae* strains UB2806(pLV10) (Table 3) or UB2806(pLV11) (data not shown) as intermediate donors. Although strains of *H. influenzae*, *N. gonorrhoeae* and *E. coli* were all tested as final recipients in no case was there evidence of mobilization of the β -lactamase plasmids by pLE2451.

Specificity of plasmid mobilization by pLE2451

We have established that although pLE2451 was not stably maintained in *E. coli*, it was capable of mobilizing pLV10 and pLV11 resident in this host. It was therefore of some interest to determine whether the conjugative plasmid has the ability to mobilize non-conjugative plasmids originally derived from enteric bacteria or from *Haemophilus* species. For this purpose several small non-conjugative plasmids were separately transformed into the restriction-deficient *E. coli* strain KH802. The resulting plasmid-bearing lines were then used as intermediate donors in triparental matings. Certain plasmids originally isolated

Table 4. Ability of pLE2451, present in initial donor *N. gonorrhoeae* KH45, to mobilize non-conjugative plasmids resident in *E. coli* KH802 to recipient *E. coli* UB1005

Intermediate donor	Transfer frequency
KH802(ColE1.Tn501)	1×10^{-4}
KH802(NTP5)	1×10^{-3}
KH802(NTP1)	$< 10^{-8}$
KH802(NTP16)	$< 10^{-8}$
KH802(RSF0885)	1×10^{-4}
KH802(pJB1)	2×10^{-3}

from *Enterobacteriaceae*, e.g. NTP5 and ColE1.Tn501 were mobilized at a frequency similar to pLV10 (Table 4). In contrast NTP1 and NTP16, which also originated in enteric bacteria, were not mobilized. This is particularly interesting since like pLV10 and pLV11 these plasmids determine the production of TEM β -lactamase. However, two further β -lactamase plasmids, RSF0885, originally isolated from *H. parainfluenzae* (Roberts *et al.*, 1977), and pJB1, originally isolated from *H. ducreyi* (Brunton *et al.*, 1979), were efficiently mobilized by pLE2451 (Table 4).

Lack of mobilization ability by H. influenzae conjugative plasmids

Several conjugative R plasmids of 30–40 megadaltons have been isolated from strains of *H. influenzae* (Saunders *et al.*, 1978). We were interested in determining whether or not there is a reciprocal relationship in plasmid mobilization between *Neisseria* and *Haemophilus* species which might account for the evolution of drug-resistance in these organisms. Accordingly, we studied the ability of three conjugative *Haemophilus* R plasmids, pRI234, pUB701 and pFR16017, to mobilize β -lactamase plasmids present in intermediate donors. These plasmids were capable of transferring themselves between *H. influenzae* strains and to *E. coli* at a frequency of about 10^{-4} . However, no mobilization of pLV10, pLV11, RSF0885 or pJB1 was detected in either intraspecific (*H. influenzae* $\times$ *H. influenzae* $\times$ *H. influenzae*) or intergeneric (*H. influenzae* $\times$ *H. influenzae* $\times$ *E. coli* or *H. influenzae* $\times$ *E. coli* $\times$ *E. coli*) matings with any of the three conjugative plasmids (data not shown).

DISCUSSION

Considerable clinical concern at the emergence of plasmid-mediated penicillin-resistance in *N. gonorrhoeae* has been heightened by demonstration of the conjugative properties of the 24.5 megadalton gonococcal plasmids. Such plasmids have been shown to promote transfer of gonococcal β -lactamase plasmids to other strains of *N. gonorrhoeae* both in vitro (Eisenstein *et al.*, 1977; Roberts *et al.*, 1977; and this paper) and in vivo (Roberts & Falkow, 1979). It is likely therefore that these 24.5 megadalton plasmids might play an important role in the evolution and spread of antibiotic resistance among pathogenic *Neisseria*. We have shown that one 24.5 megadalton plasmid, pLE2451, is highly efficient at mobilizing gonococcal β -lactamase plasmids, not only in intraspecific crosses but also in intergeneric crosses to *H. influenzae* or *E. coli*. Our results demonstrate that although pLE2451 fails to become established in *H. influenzae* or restriction-deficient *E. coli*, it does survive transiently in the latter genetic background long enough to express transfer functions necessary to mobilize resident plasmids. Transfer events involving transient vectors of this kind might be important in the wild, permitting penetration of resistance and other genes into gene pools and their subsequent dissemination in new host species. The failure of pLE2451 to promote transfer of pLV10 and pLV11 from *E. coli* or *H. influenzae* back into *N. gonorrhoeae* suggests that there is a considerable restriction barrier which limits gene flow in this direction. The relative rarity of such intergeneric transfer events would explain both the late acquisition

of antibiotic resistance plasmids by the gonococcus and their limitation to two related plasmid species. Our results suggest therefore that transfer of genes promoted by pLE2451 occurs more readily out of the gonococcus gene pool to other genera than in the reverse direction. Indeed, Laufs *et al.* (1979) have speculated that pVe445, a 4.4 megadalton plasmid found in a strain of *H. influenzae* in West Germany, may have originated naturally by mobilization of an *N. gonorrhoeae* β -lactamase plasmid by a conjugative plasmid which was not itself maintained in *Haemophilus*. The relative facility with which gonococcal β -lactamase plasmids can be mobilized in vitro to *H. influenzae* might suggest that these bacteria have restriction-modification systems in common. Indeed, the two gonococcal restriction endonucleases described thus far, *NgoI* and *NgoII* (Clanton *et al.*, 1979), are isoschizomers of *HaeII* and *HaeIII*, respectively, from *H. aegyptius*. The failure of pLE2451 to mobilize plasmids in the reverse direction might be explained if *Haemophilus* species restricted this plasmid. In addition, we have shown that recipient gonococci strongly restrict β -lactamase plasmids propagated in *Haemophilus* and other species. Despite such limitations on the intergeneric passage of pLE2451, this plasmid is capable of mobilizing indigenous *Haemophilus* non-conjugative plasmids, a property not found in conjugative plasmids isolated from this genus. Furthermore, pLE2451 is capable of efficient mobilization of several R plasmids originally isolated from enterobacteria. Roberts *et al.* (1979) found that about 15% of gonococcal strains isolated during the past 40 years harbour 24.5 megadalton plasmids. Our results demonstrate that these widely distributed plasmids are thus likely to play an important natural role in mobilizing plasmids across the species and generic boundaries between *Neisseria* and related Gram-negative bacteria.

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