Properties of Plasmids Responsible for Production of Extended-Spectrum β -Lactamases

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The extended-spectrum β -lactamases are believed to arise by mutations which alter the configuration around the active site of TEM- and SHV-type enzymes so as to increase their efficiency with otherwise nonhydrolyzable cephalosporins and monobactams. This hypothesis predicts that the genes for these new enzymes should be found on the same wide variety of plasmids that encode TEM-1, TEM-2, and SHV-1 β -lactamases and that at least some of them should be mediated by transposons. Fifteen plasmids, each encoding an extended-spectrum β -lactamase, were examined. Unlike the average TEM plasmid, all were large, ranging in size from 80 to 300 kb. All determined resistance to multiple antimicrobial agents, ranging from 5 to 11, and some conferred resistance to heavy metals and UV radiation as well. The plasmids belonged to a limited number of incompatibility (Inc) groups, including IncC, IncFI, IncHI2, and IncM. Because most of the mutations giving rise to extended-spectrum activity are G $\cdot$ C $\rightarrow$ A $\cdot$ T transitions and some of the mutant genes have as many as four base substitutions, a plasmid-determined mutator gene was searched for, but no such property was found. Several techniques were used to detect transposition of the extended-spectrum β -lactamase genes, but a mobile genetic element could not be demonstrated even though eight of the plasmids hybridized with a DNA probe derived from the *tnpR* gene of Tn3. The genesis of extended-spectrum β -lactamases may not be as simple as has been supposed.

Extended-spectrum β -lactamases are enzymes that confer resistance to cefotaxime, ceftazidime, and other broad-spectrum cephalosporins and to monobactams such as aztreonam (43). They appear to arise from genes for common plasmid-mediated SHV-1, TEM-1, and TEM-2 β -lactamases by mutations that alter the amino acid configuration around the active site of these enzymes so as to expand their spectrum of activity (19, 30, 42, 49).

TEM-1 and TEM-2 β -lactamases are determined by plasmids belonging to many different incompatibility (Inc) groups in enteric organisms (35) and are often carried on transposons (22). SHV-1 is also encoded by several plasmid types (36), but whether it is (40) or is not (33) transposon determined is in dispute.

To explore the mutation hypothesis, we have characterized plasmids responsible for extended-spectrum β -lactamase production. The aims of this study were to establish whether the plasmids belonged to many or a few Inc groups, to determine whether they carried transposons encoding the extended-spectrum activity, and to investigate whether they possessed genes facilitating the mutational alterations observed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. Table 1 lists the strains, plasmids, and phages used.

Media and resistance tests. L broth (31) was used for liquid cultures. Selection plates contained minimal medium A (15), supplemental growth factors as required, 2% agar (Difco Laboratories, Detroit, Mich.), and 0.5% glucose or lactose. The following supplements were added at the indicated concentrations (in micrograms per milliliter): L-arginine, 100; L-histidine, 28; L-leucine, 80; L-methionine, 30; L-pro-

line, 50; thiamine, 0.34; and L-threonine, 43. Antimicrobial agents were used at the indicated concentrations (in micrograms per milliliter): ampicillin, 50; gentamicin, 20; kanamycin, 25; nalidixic acid, 100; rifampin, 100; streptomycin, 25; tetracycline, 5 or 25; and tobramycin, 10.

Antibiotic resistance was evaluated by disk or agar diffusion using Trypticase soy agar plates (BBL Microbiology Systems, Cockeysville, Md.). Often a range of concentrations down to the MIC for the R⁻ host was used to detect low-level resistance (26). For selecting or scoring Hg²⁺ resistance, 0.1 mM HgCl₂ was added to appropriately supplemented minimal plates. Arsenite resistance was tested on plates containing 10 mM NaAsO₂, and tellurite resistance was scored on brain heart infusion agar (Difco) plates containing 0.5 mM K₂TeO₃ (52). UV light resistance was evaluated following growth in L broth by scoring survivors after UV exposure on L agar plates (25).

Matings. Matings and incompatibility tests were performed as previously described (44).

Gel electrophoresis. Agarose gel electrophoresis was performed by the method of Hansen and Olsen (20) or Takahashi and Nagano (53). As molecular weight standards, plasmids RP4 (54 kb) (24), R1 (92 kb) (24), *plac* (152 kb) (24), R27 (182 kb) (54), and pMG5 (420 kb) (20) were used.

Phage tests. Phages were propagated with R agar overlays on R agar plates (37). Lysates were sterilized by passage through membrane filters (pore size, 0.45 μ m; Millipore Corp., Bedford, Mass.). Spotting for phage susceptibility was performed on R agar plates. When direct spotting failed, susceptibility was tested following plasmid transfer to produce temporary derepression of pilus formation, as described by Bradley and Fleming (5). Tests with phage M (11) were performed with *Escherichia coli*, while tests with phage C1 were done after plasmid transfer to *Salmonella typhimurium* (47). Transfer deficient (Tra⁻) IncM plasmid derivatives were selected on R agar plates spread with phage M

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TABLE 1. Strains, plasmids, and bacteriophages used

Strain or phage	Relevant properties ^a	Reference
<i>Escherichia coli</i>		
AB2463	F ⁻ <i>arg his leu pro recA13 rpsL thr</i>	3
C600	F ⁻ <i>lacY leu thi thr</i>	1
HB101	F ⁻ <i>lacY leu pro recA rpsL20 thi</i>	4
JC1553	F ⁻ <i>arg his lacZ leu met recA rpsL20</i>	10
J53-2	F ⁻ <i>met pro Rif</i> ^r	12
KF641	As C600 but Nal ^r	— ^b
P90C	<i>ara Δ(lac proB)</i> _{XIII}	14
J53-2(pMG227) ^c	Plasmid encodes TEM-3	28
J53-2(pUD16)	Plasmid encodes TEM-4	41
A15R ⁺ (pMG226) ^d	Plasmid encodes TEM-6	2
BM694(pIF100)	Plasmid encodes TEM-7	19
J53-2(pMG223)	Plasmid encodes MGH-1 ^e	27
J53-2(pMG225)	Plasmid encodes YOU-1 ^f	46
J53-2(pMG224)	Plasmid encodes YOU-2 ^g	46
J53-2(R1010)	Plasmid encodes SHV-1	36
J53-2(pMG229) ^h	Plasmid encodes SHV-2	28
J53-2(pUD18)	Plasmid encodes SHV-3	39
J53-2(pUD21)	Plasmid encodes SHV-4	6
BM694(pAFF2)	Plasmid encodes SHV-5	18
J53-2(plac)	Lac ⁺ Su IncC	24
J53(R715b)	Km Sm Hg IncC	24
J53-2(R742)	Ap Km Su Tc Hg IncC	24
HB101(pOX38-Km)	Km Mob ⁻ IncFI	9
J53-1(R455)	Ap Cm Sm Sp Su Tc Hg IncFI	24
J53-2(R974)	Ap Cm Sm Su Tc Lac IncFI	36
J53(R1)	Ap Cm Km Sm Su IncFII	24
J53-2(R826)	Ap Cm Gm Km Sm Tc Hg Te IncHI2	24
J5AzR(R135)	Gm Sm Su Tc Hg IncM	24
J53(R46)	Ap Sm Sp Su Tc UV IncN	24
J53(RP4)	Ap Km Tc IncP	24
AB2463(pRB213)	Cm λ CI-P _R -Tc	3
HB101(pUCD800)	Km Tm <i>sacB</i>	16
MV10(pMK20)	Km	29
MV12(RSF1050)	pMB8::Tn3	23
CC101-106 ⁱ	F ⁺ <i>lac proB</i>	14
<i>Klebsiella pneumoniae</i>		
CF104	Contains pCFF04 encoding TEM-3	8
CF504	Contains pCFF14 encoding TEM-5	8
2633E	Contains pMG228 encoding TEM-9	51
CF704	Contains pCFF34 encoding CAZ-2	8
<i>Salmonella typhimurium</i> LT2	<i>leu50</i>	
Bacteriophages		
C1		47
M		11

^a Plasmid resistance abbreviations: Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Hg, mercuric chloride; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Su, sulfonamide; Tc, tetracycline; Te, potassium tellurite; Tm, tobramycin; Tp, trimethoprim; UV, UV radiation.

^b From D. Hooper.

^c Plasmid not named in original publication. Originated in *K. pneumoniae* 86-15 (28).

^d Plasmid not named in original publication. Originated in *E. coli* HB 251 (2).

^e MGH-1 β -lactamase is indistinguishable from TEM-10 (45) in isoelectric point (pI) (5.57) or reactions with inhibitors (46) but has been given a distinct name until its sequence identity has been established.

and containing ampicillin to prevent simple loss of the resident plasmid. Surviving colonies were screened for lack of resistance transfer.

Mutator detection. Plasmids were transferred to strains CC101 to CC106, which carry different mutations at the same coding position of the *lacZ* gene such that each reverts via one of the six possible base substitutions (14). Lac⁺ revertants were selected on lactose minimal medium. Fifteen seconds of UV light exposure was used to stimulate mutations.

tnpR hybridization. The 295-bp *Bam*HI-*Hae*II fragment internal to the *tnpR* gene of Tn3 carried by RSF1050 was used as a *tnpR* probe (23). Restriction fragments were separated by gel electrophoresis using low-melting-point agarose. The band of interest was cut from the gel, extracted with GeneClean (Bio 101 Inc., La Jolla, Calif.), and labeled with digoxigenin using the Genius Kit from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Plasmids encoding extended-spectrum β -lactamases were separated by agarose gel electrophoresis and transferred to nitrocellulose or nylon membranes by blotting (50). The standard protocol specified with the Genius Kit provided stringent conditions for hybridization. Hybrids were detected using an antidigoxigenin-alkaline phosphatase conjugate and a phosphatase substrate that was either chromogenic or chemiluminescent (Southern-Light, Tropix, Inc., Bedford, Mass.).

Transposon detection. (i) **Gene amplification.** Extended-spectrum β -lactamase plasmids were transferred to *recA E. coli* HB101 containing the multicopy 4.1-kb plasmid pMK20. Since transposition from a low- to high-copy-number plasmid increases β -lactamase expression, clones more resistant to ampicillin or ceftazidime were selected and screened by agarose gel electrophoresis for a change in size of the pMK20 plasmid band (33).

(ii) **Blocked transfer.** Rec⁻ strains were constructed containing the test plasmid and one proficient for transfer under conditions in which transfer of ampicillin resistance by the first plasmid was blocked. The barriers to transfer were host range for those plasmids observed not to transfer from *E. coli* to *S. typhimurium*, mutations selected with phage M that rendered IncM plasmid genetically Tra⁻, and a temperature of 42°C which made IncHI2 plasmids phenotypically Tra⁻. The proficient plasmid in each case was the F factor derivative pOX38-Km, which transferred at high frequency under all conditions. Resistance to at least one other antibiotic carried by the test plasmid was selected in addition to ampicillin resistance to detect mobilization of the plasmid as an independent replicon. A Tra⁻ derivative of RP4 carrying TnI was included as a positive control.

(iii) **Gene inactivation.** Extended-spectrum β -lactamase plasmids not conferring resistance to kanamycin or tobramycin were transferred to *recA E. coli* HB101 containing pUCD800. Cells containing pUCD800 cannot grow on 5% sucrose unless the plasmid *sacB* gene is inactivated, as by transposon insertion (16). Accordingly, survivors were se-

^f YOU-1 β -lactamase also has a pI of 5.57 but differs from TEM-10 and MGH-1 in inhibitor profile (46).

^g YOU-2 β -lactamase has a pI of 5.2 and an inhibitor profile indistinguishable from that of TEM-101 (46).

^h Plasmid not named in original publication. Originated in *K. pneumoniae* 86-1 (28).

ⁱ A set of six P90C derivatives carrying different *lacZ* mutations affecting residue 461 on F⁺*lac proB* episomes such that only a particular base substitution will restore the Lac⁺ phenotype (14).

TABLE 2. Properties of plasmids encoding extended-spectrum β -lactamases

β -Lactamase	Plasmid	Additional resistances ^a	Size (kb)	Inc group
TEM-3	pMG227	Km Su Tc Tm Hg	85	M
TEM-3	pCFF04	Ak Km Nm Su Tc Tm Hg	80	M
TEM-4	pUD16	Gm Km Nm Sm Sp Su Tc Tm Tp UV	190	C
TEM-5	pCFF14	Km Nm Sm Su Tc	185	FI
TEM-6	pMG226	Cm Km Nm Sm As Te UV	240	HI2
TEM-7	pIF100	Ak Gm Km Su Tc Tm Hg	90	M
TEM-9	pMG228	Cm Gm Sm Sp Su Tc Tm Tp	190	C ^b
MGH-1	pMG223	Ak Cm Gm Km Nm Sm Sp Su Tc Tm Tp Te UV	285	HI2
YOU-1	pMG225	Cm Gm Km Nm Sm Sp Su Tc Tm Tp Hg Te UV	240	HI2
YOU-2	pMG224	Cm Gm Km Nm Sm Sp Su Tc Tm Tp Hg Te UV	260	HI2
CAZ-2	pCFF34	Ak Km Nm Su Tc Tm Hg	80	M
SHV-2	pMG229	Sm Sp Su Tc Tp	205	C
SHV-3	pUD18	Ak Gm Km Nm Sm Sp Su Tc Tm Tp As	300	FI ^c
SHV-4	pUD21	Ak Gm Km Nm Sm Sp Su Tc Tm Tp As	275	FI ^c
SHV-5	pAFF2	Gm Sm Sp Su Tc	95	M

^a Resistance abbreviations are as in footnote *a* of Table 1 plus the following: Ak, resistance to amikacin; As, resistance to sodium arsenite; Nm, resistance to neomycin.

^b Provisional assignment since entry exclusion with a recipient carrying an IncC plasmid was so complete that incompatibility per se could not be tested.

^c Provisional assignment because of unusually large plasmid size and nonreciprocal incompatibility behavior.

lected on Trypticase soy agar plates containing 5% sucrose and kanamycin or tobramycin to prevent loss of pUCD800.

The single plasmid (pMG226) not encoding resistance to tetracycline was transferred to *recA* *E. coli* AB2463 carrying pRB213. Plasmid pRB213 carries a cryptic tetracycline resistance gene under the control of a lambda phage repressor, such that resistance is not expressed (3). Plasmids with inactivating insertions into the lambda *cI* gene were selected on Trypticase soy agar plates containing 5 μ g of tetracycline per ml and sized by agarose gel electrophoresis. Strain AB2463(pRB213) containing R1 (Tn3) served as a positive control.

RESULTS

Properties of plasmids. Fifteen plasmids encoding extended-spectrum β -lactamases were transferred to *E. coli* J53-2 as a common host and tested for resistance to a variety of antimicrobial agents and mercuric chloride, potassium tellurite, sodium arsenite, and UV light. Table 2 shows that all the plasmids determined resistance to multiple agents in addition to β -lactams. Fourteen of the plasmids encoded resistance to tetracycline, 14 encoded resistance to sulfonamide, 12 encoded resistance to kanamycin, 11 encoded resistance to streptomycin, 11 encoded resistance to tobramycin, 10 encoded resistance to neomycin, 9 encoded resistance to spectinomycin, 8 encoded resistance to gentamicin, 8 encoded resistance to trimethoprim, 6 encoded resistance to amikacin, and 5 encoded resistance to chloramphenicol. In addition, five protected against UV radiation, six conferred resistance to mercuric chloride, four gave resistance to potassium tellurite, and three provided resistance to sodium arsenite. In addition to β -lactam resistance, the

fewest other resistances encoded on a plasmid was 5 and the most was 13.

As estimated by agarose gel electrophoresis, plasmids determining extended-spectrum enzymes ranged in size from 80 to about 300 kb (Table 2).

Plasmids of this size belong to relatively few Inc groups. To reduce the number of incompatibility crosses required to identify the groups, preliminary phage tests were performed. *E. coli* J53-2 derivatives were spotted with bacteriophage M, a donor-specific phage lytic for strains carrying IncM plasmids (11). Strains carrying pCFF04, pIF100, pCFF34, and pAFF2 were directly phage M susceptible, while pMG227 was lysed when temporarily derepressed for pilus production on transfer to a new host (5). In confirmation of this assignment, pMG227, pCFF04, pIF100, and pCFF34 proved incompatible with IncM plasmid R135. Plasmid pAFF2 was expected to belong to the IncM group since it was derived by recombination between a Tra⁻ plasmid carrying the SHV-5 gene and IncM plasmid R135 (18).

Phage C1, a donor-specific phage active on plasmids of IncC in salmonellae (47), was used to screen for plasmids of this Inc group. Only some of the remaining plasmids of unknown Inc specificity could be transferred by conjugation to *S. typhimurium*, but pMG229 conferred susceptibility to phage C1. To evaluate other plasmids, they were transferred to *E. coli* KF641 carrying IncC plasmid *plac*, which causes the chromosomally Lac⁻ bacterial host to produce red colonies on MacConkey indicator medium unless *plac* is eliminated. Transfer of plasmids pUD16 and pMG229 into this strain produced white Lac⁻ colonies, indicating that these plasmids belong to the IncC group. Transfer of plasmid pMG228 into KF641(*plac*) could not be detected, nor could kanamycin resistance mediated by IncC plasmids R715b or R742 be transferred into *E. coli* J53(pMG228) despite ready transfer into R⁻ J53. These findings indicate marked entry exclusion between pMG228 and IncC plasmids and suggest that pMG228 also belongs to the IncC group.

Plasmid pCFF14 was assigned to IncFI by transfer to *E. coli* KF641 carrying IncFI plasmid R974, which also carries a *lac* gene. Transconjugants became Lac⁻ and lost resistance to chloramphenicol determined by R974. In the reciprocal cross with R974 as the entering plasmid, kanamycin resistance determined by pCFF14 was retained; however, another IncFI plasmid, R455, was incompatible with pCFF14 whichever was the resident plasmid. Plasmids pUD18 and pUD21 carried identical resistances, except for their SHV-type β -lactamase genes, and behaved similarly in compatibility tests. Both were somewhat temperature sensitive for transfer, with a conjugation frequency 20-fold higher at 26°C than at 37°C. As the entering plasmid pUD18 or pUD21 was incompatible with R455 or R974, but when R455 or R974 was introduced into a recipient carrying pUD18 or pUD21, they coexisted stably. This nonreciprocal incompatibility behavior is not unusual for IncFI plasmids (21) but together with the unusually large size of pUD18 and pUD21 has led to their being only provisionally assigned to the IncFI group (Table 2).

Four of the plasmids encoded resistance to potassium tellurite, a property virtually confined to plasmids of the H Inc group in *E. coli* (54). IncHI plasmids are also thermosensitive for transfer. Plasmids pMG223, pMG224, pMG225, and pMG226 all transferred at frequencies of about 10⁻⁴ per donor after overnight mating at 30°C but at frequencies of 10⁻⁷ or less when mating was done at 43°C. On direct testing, pMG226 proved to be incompatible with IncHI2 plasmid R826. The number of antibiotic resistance markers

TABLE 3. Plasmid mutator effects

Plasmid	UV treatment	No. of Lac ⁺ colonies/10 ⁸ cells					
		A · T→C · G ^a	G · C→A · T	G · C→C · G	G · C→T · A	A · T→T · A	A · T→G · C
R ⁻	—	1	3	0	0	0	1
	+	2	5	0	1	6	5
pCFF04	—	3	0	0	0	2	2
pUD16	—	4	2	0	2	0	2
	+	18	80	5	7	37	20
pMG226	—	0	1	0	0	0	0
	+	9	144	1	5	70	88
pMG228	—	7	2	0	2	0	3
pMG224	—	0	1	0	2	0	0
	+	10	64	4	4	30	39
pAFF2	—	0	1	0	2	0	0
R46	—	2	2	0	0	0	0
	+	26	241	8	31	106	160

^a Base substitution required to restore Lac⁺.

carried by pMG223, pMG224, and pMG225 made establishing their incompatibility specificity difficult until it was noted that pMG226 determined resistance to sodium arsenite while the other plasmids did not. Transfer of pMG223, pMG224, or pMG225 into a recipient carrying pMG226 resulted in loss of arsenite resistance, proving that all four plasmids belong to the IncHI2 group.

Plasmid mutator activity. UV protection is a relatively uncommon attribute, yet it was present on 6 of the 15 plasmids studied here. UV protection results from increased error-prone repair of UV-damaged DNA and consequently enhances UV mutagenesis. In addition, some UV resistance plasmids have a separate gene that contributes to the mutator effect (38).

To evaluate such a mutator activity, extended-spectrum β -lactamase plasmids were transferred to a set of *E. coli* strains developed by Cupples and Miller (14) that were deleted for chromosomal *lac proB* genes and carried F'*lacI lacZ proB*⁺ episomes, with each *lacZ* due to an allele that could be corrected only by a specific base substitution. The rate of reversion to Lac⁺ in such strains was thus a measure of the frequency of particular base mutations. Plasmid R46 was included as a control, since it is known to have mutator effects (38).

As shown in Table 3, none of the plasmids tested enhanced the basal frequency of base substitution mutations. Even R46 had no effect in this system unless the strains were given a short exposure to UV light. Similarly, UV^r plasmids pUD16, pMG224, and pMG226 had a UV-dependent mutator effect, but they produced no increase in the frequency of base substitutions without UV. Furthermore, even after UV exposure no mutator activity could be demonstrated with other extended-spectrum β -lactamase plasmids that lacked a UV^r gene (data not shown).

Transposon carriage. To test for the physical presence of DNA sequences related to transposability, a *Bam*HI-*Hae*II fragment of RSF1050 internal to the *tnpR* gene of Tn3 was tested as a probe with plasmid DNA immobilized on filters. Hybridization was observed under stringent conditions with plasmids pMG227, pCFF04, pMG226, pUD16, pIF100, pMG228, pMG224, and pCFF34 and R1 (containing Tn3) or RP4 (containing Tn1) as positive controls but not with pCFF14, pMG223, pMG225, or plasmids carrying SHV-type β -lactamase genes.

Three techniques were used to detect transposition of extended-spectrum β -lactamase genes. The first relied on the

higher level of β -lactam resistance produced when the gene copy number has increased on transposition from a low- to high-copy-number plasmid (33). Plasmids in Table 2 (except pMG228, pMG224, pUD18, and pAFF2) were transferred by conjugation to RecA⁻ strain *E. coli* HB101 containing multicopy plasmid pMK20. Colonies selected on a β -lactam concentration above the MIC for the parent plasmids were screened by agarose gel electrophoresis for the increased size of a pMK20 transposon derivative. None were found, although with the same system transposition from R1 or RP4 to pMK20 could easily be detected (33).

The second procedure looked for transmissibility of β -lactam resistance from a strain containing two plasmids, one proficient for transfer and the other not self-transmissible but carrying a β -lactamase gene. Of several plasmids tested as potential transposon targets, pOX38-Km derived from the F plasmid (9) provided the highest transposon yield in control experiments. Three selections against transfer of the β -lactamase plasmid were employed. Some plasmids transferred poorly or not at all between *E. coli* and *S. typhimurium*. Accordingly, *E. coli* HB101 strains containing pOX38-Km and plasmid pUD16 or pMG228 were crossed with *S. typhimurium* LT2, selecting for transfer of ampicillin or ceftazidime resistance, but no transposon derivatives were found. For IncH plasmids pMG223, pMG224, and pMG225, temperature was used to block transfer. HB101 derivatives carrying one of these plasmids and pOX38-Km were mated with RecA⁻ strain JC1553 at 43°C, selecting for transfer of ampicillin or ceftazidime resistance. Again, no transposition to pOX38-Km was detected. Finally, phage M was used to select Tra⁻ derivatives of plasmids pCFF04, pIF100, and pCFF34. The Tra⁻ derivatives were introduced into HB101 containing pOX38-Km by transformation and crossed with JC1553, selecting ampicillin or ceftazidime resistance. With a Tra⁻ derivative of RP4 carrying Tn1, transposition to pOX38-Km was readily detected but it was not found with Tra⁻ plasmids encoding extended-spectrum β -lactamases.

The third procedure relied on insertional inactivation of a gene producing a lethal product. Cells containing pUCD800 are unable to grow on media with 5% sucrose unless the *sacB* gene carried by the plasmid is inactivated, as by transposition of DNA from a second coexisting plasmid (16). Since pUCD800 carries resistance to kanamycin or tobramycin, simple loss of the plasmid was prevented by including one of these aminoglycosides in the medium. Consequently, the test was limited to those extended-spectrum β -lactamase

plasmids that did not themselves confer kanamycin or tobramycin resistance. *E. coli* HB101 derivatives carrying pUCD800 and pCFF14, pMG229, or pAFF2 were constructed and plated on media with 5% sucrose and 10 µg of tobramycin per ml, but no colonies derived by transposition into the *sacB* gene were found. The final test for transposition utilized plasmid pRB213, which carries a cryptic tetracycline resistance gene that is under the control of a phage lambda repressor. Insertions into the repressor gene allow expression of tetracycline resistance. Unfortunately, all but one of the extended-spectrum β-lactamase plasmids carried tetracycline resistance, but plasmid pMG226 did not. pMG226 and control plasmid R1 carrying Tn3 were transferred to a Rec⁻ strain carrying pRB213. With R1, Tc^r colonies appeared at a frequency of 8×10^{-6} , while none (frequency, $<5 \times 10^{-8}$) were obtained with pMG226, indicating that this selection also failed to detect transposition.

DISCUSSION

If extended-spectrum β-lactamases arise simply by mutation, their distribution on plasmids should reflect that of their TEM-1, TEM-2, and SHV-1 parents and at least some of them should be carried by transposons. We found that 15 plasmids encoding various extended-spectrum β-lactamases were conspicuous for their large size (80 to 300 kb) and multiplicity of resistance determinants in addition to β-lactamase. This is not typical of the distribution of TEM-type β-lactamase in nosocomial isolates of *E. coli*, in which TEM is most often carried along with only a few other resistance genes by plasmids that are considerably smaller than 80 kb (13). The Inc specificity of the plasmids was challenging to investigate because their multiresistance often made suitable test plasmids without overlapping markers difficult to find. Test plasmids carrying a *lac* gene proved useful, as did such properties as donor-specific phage sensitivity and carriage of tellurite resistance, which are uniquely associated with a particular Inc group (54). Several of the plasmids had previously been assigned to IncM (8) or IncC (41) groups. Table 2 shows that extended-spectrum β-lactamase plasmids also occur in the IncFI and IncHI2 groups. Considering the variety of plasmid types encoding TEM-1, TEM-2, and SHV-1, the distribution of extended-spectrum β-lactamases seems limited and raises questions of whether certain plasmid types are more successful in a nosocomial environment or have properties that promote resistance gene evolution.

The *bla*_{tem} and *bla*_{shv} mutations found to date are mainly G → A · T transitions (34). Furthermore, sequence analysis has shown as many as four differences between native and mutant genes (34). These findings suggested that a mutator facilitating specific base substitutions might be a feature of these plasmids, especially since some provided UV protection which can be associated with mutator properties (38). Using the sensitive detection system developed by Cupples and Miller (14), the only mutation enhancement that could be detected was that known to occur with UV^r plasmids after UV exposure. While the existence of a mutator function carried by the bacterial host has not been excluded, there are several ways that multiple point mutations could arise without invoking the phenomenon of directed mutation (7). In particular, Lenski et al. (32) have shown how the incorporation of sequential mutations is favored by a selective advantage of the intermediate genotype. The benefits of individual β-lactamase mutations (48) thus encourage their accumulation at what might otherwise seem an unlikely frequency.

Carriage of at least some of these extended-spectrum β-lactamases by a transposon was anticipated but could not be demonstrated. Since this difficulty was unexpected, several techniques were used to evaluate transposability, with consistently negative results. Most TEM-type extended-spectrum β-lactamase plasmids hybridized with a DNA probe specific for the *tnpR* gene of TnA, so that at least part of one essential component of the transposon machinery is present. Whether other components are deleted or defective remains to be established. Even *bla*_{tem-1} is not always transposable. Gerlach and Wiedemann found that only 50% of TEM-1-encoding plasmids from *E. coli* could transpose ampicillin resistance (17). Since their spread would be facilitated, it is likely that in time other isolates of extended-spectrum enzymes will be found on transposons.

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