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Transfer of a Plasmid-Specified Beta-Lactamase Gene from *Haemophilus influenzae*

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A number of ampicillin-resistant strains of *Haemophilus influenzae* could donate a gene specifying the type IIIa (TEM) β-lactamase to *Haemophilus parainfluenzae*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Donor strains rapidly lost their ability to transfer ampicillin resistance on storage or subculture. Such strains also apparently contained a single species of covalently closed circular deoxyribonucleic acid of contour length 1.2 μm, equivalent to about 2.5 × 10^6 daltons. No species of plasmid deoxyribonucleic acid large enough to encode sex factor activity was detected. Despite this, transfer occurred to several bacterial genera in the presence of deoxyribonuclease, suggesting that transmissibility was by conjugation. The β-lactamase gene was generally unstable after transfer and was lost in the absence of selection. Where stable transcipients were found, this was evidently by insertion of the β-lactamase gene into the host chromosome. In *P. aeruginosa* insertion was always accompanied by induction of auxotrophy for adenine, suggesting insertion at a specific site. It is believed that insertion also occurred at one site on the chromosome of *Escherichia coli*. Crypticity measurements for β-lactamase activity showed that there was little or no penetration barrier to β-lactam drugs in *Haemophilus*. This may explain the long delay in the acquisition of ampicillin resistance by this organism.

Ampicillin-resistant strains of *Haemophilus influenzae* have become increasingly common in recent years, thus contradicting the previously held assumption that the *H. influenzae* population was uniformly susceptible to this antibiotic. Resistance in all cases so far examined (8, 9, 15, 18, 25, 27) is due to the acquisition by *H. influenzae* of a gene specifying the TEM or type IIIa β-lactamase (22). This gene can form part of the translocatable genetic element transposon A (TnA) (11, 12), which is borne on a range of physically and genetically distinct plasmids found in a variety of bacterial species (13).

Transferable kanamycin resistance has been reported in *H. influenzae* (6). Furthermore, transfer of ampicillin resistance between strains of this organism, but not to *Escherichia coli*, has also been described by Thorne and Farrar (26). We report the transfer of a gene specifying the type IIIa β-lactamase from ampicillin-resistant strains of *H. influenzae* to *H. parainfluenzae*, *E. coli*, and *Pseudomonas aeruginosa*. The behavior and expression of this gene in these bacterial species is described together with possible implications for the spread of drug resistance to previously susceptible strains of *Haemophilus*.

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MATERIALS AND METHODS

[2-^{3}H]adenine was obtained from the Radiochemical Centre, Amersham; pancreatic deoxyribonuclease (DNAse) I was from Sigma Chemical Co., London. All other materials have been described previously (3, 10, 25).

Bacterial strains used. See Table 1.

Growth and storage of bacteria. All organisms were grown in brain heart infusion (Difco), supplemented with 5% (vol/vol) Fildes peptic digest (Oxoid) for the growth of *Haemophilus* organisms (BHF). Stock cultures were subcultured in BHF immediately on receipt and stored at −70°C. Where required, liquid medium was solidified by the incorporation of 1.2% (wt/vol) agar (Difco). *Haemophilus* strains were generally grown in an atmosphere supplemented with 5 to 10% CO_2. Where required, antibiotics were incorporated at the following concentrations: ampicillin, 20 μg/ml; carbenicillin, 500 μg/ml; rifampin, 100 μg/ml; streptomycin, 500 μg/ml; and tetracycline, 100 μg/ml.

Detection of β-lactamase. β-Lactamase-producing strains of *E. coli* and *P. aeruginosa* were detected by spotting the chromogenic cephalosporin 87/312 on colonies growing on solid media. Formation of a red color indicated the presence of a β-lactamase.

Suspensions of *H. influenzae* were prepared by

TABLE 1. *Bacterial strains used*[a]

Species	Designation	Genetic markers	Origin
Haemophilus influenzae	23142	*amp*^{r}	C. Thornsberry
	2486	*amp*^{r}	P. Cavanagh
H. parainfluenzae	28366		P. Cavanagh
Escherichia coli	W3110		Ref. 10
	W3110 (R2486)	W3110 × 2486	This paper
	UB1139 (pUB306)	*thy*$^-$ *leu*$^-$ *met*$^-$ *nal*^{r} RP1—ampicillin susceptible	Ref. 3 and 5
	J5-3 (pUB307)	*pro*$^-$ *met*$^-$ *nal*^{r} RP1—TnA deleted	P. M. Bennett
Pseudomonas aeruginosa	Ps18	*rif*^{r}	Ref. 10
	PAO8	*str*^{r} *met*$^-$ *ilv*$^-$	V. Stanisich
	Ps18 (R23142)	*rif*^{r} *amp*^{r} (Ps18 × 23142)	This paper

[a] Abbreviations: *amp*^{r}, *nal*^{r}, *rif*^{r}, *str*^{r}, resistance to ampicillin, nalidixic acid, rifampcin, and streptomycin, respectively. RP1 specifies resistance to ampicillin, tetracycline, and neomycin/kanamycin.

picking several colonies of the test organism from a fresh overnight culture and emulsifying in physiological saline (0.5 ml). Samples (50 μl) of the cell suspensions were distributed into the wells of a Cooke microtiter plate that contained 50 μl of an aqueous solution of 87/312 (500 μg/ml in 0.1 M phosphate buffer, pH 7.0). A color change from yellow to red indicated the presence of β-lactamase.

Preparation of enzymes. Enzymes were prepared as described in reference 25.

β-Lactamase assays. All assays were performed iodometrically by the method of Perret (20) as modified by Novick (19). Ampicillin was used as substrate at a concentration of 6 mM, and assays were performed at pH 7.0 and 37°C. Enzyme units are defined as micromoles of substrate hydrolyzed per minute.

Crypticity measurements. These were determined by incubation of ampicillin with washed cells of the relevant organism (i) intact and (ii) sonicated (for 3 min at 37°C). Estimation of the amount of substrate destroyed in each instance gives a measure of the available β-lactamase activity. Crypticity = (specific enzyme activity of broken cells)/(specific enzyme activity of whole cells).

Genetic transfer. Exponential-phase cultures of donor strains were mixed 1:10 with recipient strains, and incubation continued at 37°C without shaking for 4 h. In some cases, pancreatic DNase I was included in the mating mixture at a concentration of 20 μg/ml. Mating mixtures were Vortex-mixed at the end of the mating period, and dilutions were plated on appropriately supplemented agar.

Elimination of drug resistance. Organisms were grown in the presence of ethidium bromide (2 μg/ml) for 18 h at 37°C. Dilutions of the culture were then plated on drug-free BHF medium and incubated for 18 h at 37°C. Suitable plates were then replicated onto similar medium containing ampicillin.

Isolation of plasmid deoxyribonucleic acid (DNA). *P. aeruginosa* Psa18 (R23142) was labeled with [^{3}H]adenine (5 μCi/ml; 5 μg/ml). All other bacteria were labeled with [^{3}H]thymidine, lysed, and analyzed by ethidium bromide-CsCl gradient centrifugation as described previously (10).

Electron microscopy of DNA. Preparations of plasmid DNA were examined by electron microscopy as described in reference 3.

TABLE 2. *Transfer of type IIIa β-lactamase gene from Haemophilus influenzae*

Donor	Recipient	Transfer frequency[a]	
		In absence of DNase	In presence of DNase
H. influenzae 23142	*H. parainfluenzae*	1×10^{-6}	3×10^{-6}
	E. coli W3110	8×10^{-5}	7×10^{-5}
	P. aeruginosa Ps18	3×10^{-5}	2×10^{-5}

[a] Transfer frequencies were determined by dividing the number of ampicillin-resistant recipients present at the end of the mating by the number of donor cells present at the beginning of mating.

RESULTS

Transferability of ampicillin resistance from *H. influenzae*. All ampicillin-resistant strains of *H. influenzae* examined here contain a type IIIa β-lactamase (25). Because the gene specifying this enzyme is commonly found on R factors (22), attempts were made to transfer ampicillin resistance to other bacteria. The strains used could indeed transfer resistance to a variety of organisms (Table 2). Donor ability, but not resistance, declined rapidly on storage or subculture. However, strains that had apparently lost their ability to transfer directly to *E. coli* could do so indirectly if an intermediate *H. influenzae* donor strain was used in a triparental cross. To eliminate the possibility of gene transfer by transformation, matings were re-

peated in the presence of DNase (Table 2). No appreciable difference in transfer rate could be determined in the presence or absence of DNase.

The β-lactamase gene was highly unstable when transferred to *H. parainfluenzae*. The presence of the gene could, however, be detected readily in unstable recipient strains by the analytical isoelectric-focusing technique as previously described (25). Direct transfers from *H. influenzae* to *E. coli* were again characterized by instability of the β-lactamase gene. However, transfers either directly to *P. aeruginosa* or by triparental matings to *E. coli* always resulted in stable transcipients. In all cases, furthermore, the β-lactamase gene was not further transferable from such stable transcipients to other strains.

Properties of extrachromosomal DNA in strains carrying the β-lactamase gene. All strains of *H. influenzae* used that were capable of acting as donors of the β-lactamase gene contained covalently closed circular DNA amounting to about 2% of the chromosomal DNA as judged by ethidium bromide-CsCl gradient centrifugation (Fig. 1). Donor strains of *H. influenzae* could be cured of ampicillin resistance by growth in medium containing ethidium bromide (Table 3). Cured strains contained no extrachromosomal DNA (e.g., Fig. 1). Concomitant loss of this DNA and resistance shows that the β-lactamase gene is plasmid linked. Circular DNA in donor strains had a contour length of about 1.2 ± 0.02 μm, equivalent to a molecular weight of about 2.5 Mdal (17) (Fig. 2). No larger plasmid DNA molecules were detected in any donor strain. In addition, transcipients from crosses either to *E. coli* or *P. aeruginosa* that had received the β-lactamase gene contained no detectable extrachromosomal DNA.

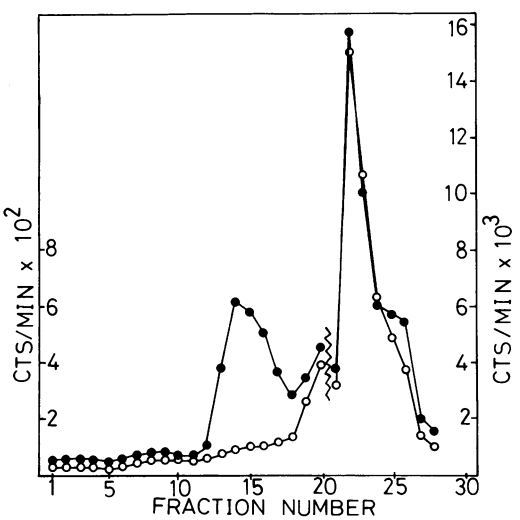

FIG. 1. *Ethidium bromide-CsCl density gradient centrifugation of DNA from strains of H. influenzae. Radioactive cultures were lysed and analyzed separately on ethidium bromide-cell gradients. Symbols: (●) DNA from strain 2486 (ampicillin resistant); (○) DNA from strain UB 2824 (ampicillin-susceptible variant of 2486). Note the change of scale between fractions 20 and 21 in each case.*

Properties of strains of *E. coli* and *P. aeruginosa* receiving the β-lactamase gene. All strains of *P. aeruginosa* tested (30 clones from three separate matings) that had received the β-lactamase gene from *H. influenzae* were found to have become auxotrophic for adenine. Since these strains contain no extrachromosomal DNA (see above), this is probably due to insertion of the β-lactamase gene into the chromosomes of *P. aeruginosa*, causing disruption of one of the two loci known for adenine production in this organism (14). Detailed mapping of the site of insertion was precluded because the strains of *P. aeruginosa* used would not receive the *Pseudomonas* sex factor FP2 (J. R. Saunders, unpublished data).

No equivalent induction of auxotrophic mutations was observed in *E. coli* strains that had received the β-lactamase gene from *H. influenzae*. However, preliminary mapping experiments place the site of insertion of the β-lactamase gene close to *trp* on the chromosome of *E. coli* (J. R. Saunders, unpublished data).

Attempts were made to mobilize the TEM β-lactamase gene from the insertion site in the chromosomes of these two organisms. This was done by infecting such strains with derivatives of the R factor RP1 that no longer specify β-lactamase. These were pUB 306, a derivative of RP1 that contains point mutations both in the type IIIa β-lactamase gene and in the gene specifying intrinsic resistance to penicillins (5; N. Curtis, personal communication), and pUB 307, a variant of RP1 in which the whole of TnA, and hence the β-lactamase gene, has been deleted (23). Strains of *P. aeruginosa* and *E. coli* were maintained for about 20 generations in the absence of drug selection and then tested for transfer ability. Neither R factor was able to mobilize the inserted β-lactamase gene (Table

TABLE 3. *Curing of ampicillin resistance from Haemophilus influenzae*

Strain	Total colonies tested	Ampicillin-susceptible colonies
23142	690	5
2486	403	4

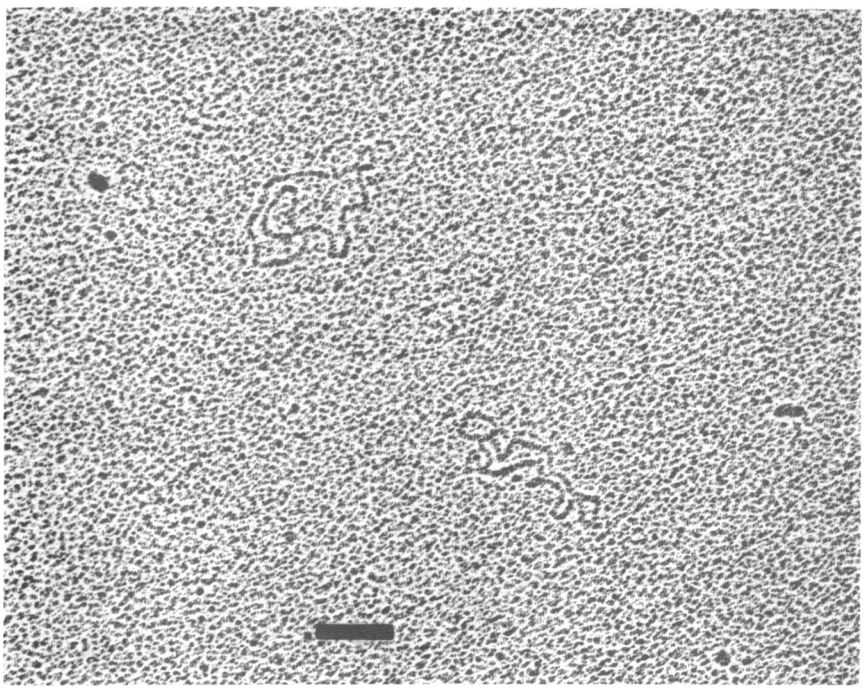

FIG. 2. *Electron micrograph of extrachromosomal DNA from H. influenzae 2486. Bar represents 0.1 μm.*

4) in either species, although the R factors were evidently capable of transferring themselves. Furthermore, the presence of either R factor did not promote the reversion of *P. aeruginosa* to adenine prototrophy.

β-Lactamase production and crypticity values. Both donor and transcipient strains produced similar levels of cell-free β-lactamase (Table 5), as did ampicillin-resistant strains that had lost transfer ability (data not shown). However, hydrolysis of ampicillin by whole-cell preparations varied markedly. Whole-cell cultures of the *Haemophilus* strains hydrolyzed ampicillin at a similar rate to the cell-free extracts. In the case of the *E. coli* and *Pseudomonas* transcipients, whole-cell cultures were much less efficient at hydrolyzing the substrate than their corresponding cell-free enzyme preparations (Table 5).

DISCUSSION

We have demonstrated that the ampicillin resistance found in the *H. influenzae* strains studied here is plasmid linked. Resistance is due to the type IIIa β-lactamase and is specified by an extrachromosomal element of about 2.5 Mdal. We proposed to call this plasmid pUB702. Assuming that the amount of covalently closed circular DNA present in resistant strains (about 2% of the chromosomal DNA) represents

TABLE 4. *Transfer of inserted β-lactamase gene by derivatives of RP1* [a]

Plasmid	Selection on:	Transfer frequency [a]	
		From *P. aeruginosa* Ps18 R23142	From *E. coli* W3110 (2486)
pUB306	Tetracycline (100 μg/ml)	3×10^{-4}	5×10^{-4}
	Carbenicillin (500 μg/ml)	$<10^{-10}$	$<10^{-10}$
pUB307	Tetracycline (100 μg/ml)	1×10^{-4}	3×10^{-4}
	Carbenicillin (500 μg/ml)	$<10^{-10}$	$<5 \times 10^{-9}$

[a] The recipient in all cases was *P. aeruginosa* PAO8, and counterselection was by streptomycin (500 μg/ml).

TABLE 5. *β-Lactamase activity of cell-free preparations and crypticity values*

Organism	Enzyme units/mg of protein	Crypticity value
Haemophilus influenzae 2486	0.03	1
Escherichia coli W3110 (R2486)	0.035	30
H. influenzae 23142	0.03	1
Pseudomonas aeruginosa (R23142)	0.03	40

the lower limit of plasmid DNA and that the molecular weight of the *H. influenzae* chromosome is 1.7×10^9 (7), then this plasmid is present at about 14 copies per chromosome equivalent. The molecular properties of pUB702 are closely similar to those of the smaller (3 Mdal) of two plasmid species, named RSF0885, isolated by Falkow and co-workers (7, 8) from a series of ampicillin-resistant *H. influenzae*. Our strains were capable of donating the TEM β-lactamase gene to other bacteria even in the presence of DNase, thus eliminating transformation as the mechanism of transfer. The ability to transfer to such widely differing organisms as *H. parainfluenzae*, *E. coli*, and *P. aeruginosa* tends also to rule out transduction. It seems more likely that transfer was by conjugation. It is therefore surprising that no plasmid species large enough to encode sex factor activity were found. This may be because such strains contained a sex factor not detected by the methods used, or alternatively lost from the cells relatively rapidly. Support for the latter explanation comes from the observation that donor ability but not β-lactamase production was lost rapidly. Transfer of resistance could therefore have occurred via a plasmid coaggregate (4) or class II transfer system (1), involving an unstable sex factor. An alternative explanation that we cannot rule out is that the donor strains originally contained pUB702 plus another unstable self-transmissible plasmid carrying an identical β-lactamase gene.

Although the TEM β-lactamase gene could be easily transferred to other bacterial species, it was generally unstable in *H. parainfluenzae* and *E. coli*. Similarly, Elwell et al. (8) found that RSF 0885 was unstable when transformed into *E. coli*. Despite this general instability, we could find stable transcipients especially in *P. aeruginosa*, where all such strains were highly stable. Stability was evidently due to insertion at specific sites in the chromosomes of *P. aeruginosa* and *E. coli*. This is in line with the transposable nature of the gene specifying the TEM β-lactamase (3, 11, 12). Generally, transposons can be repeatedly translocated to other genomes (11, 12). This was not the case with the inserted β-lactamase gene studied here, which could not be mobilized by R factors known to accept TnA. De Graaff et al. (7) have shown that the RSF 0885 contains only about one-third of the base sequences found in TnA. Assuming that pUB702 and RSF 0885 are closely similar, insertion of the β-lactamase gene into the genomes of these two bacterial species may therefore have been by normal recombination with pUB702 rather than by transposition per se.

The induction of auxotrophic mutations in *P. aeruginosa* by insertion of the β-lactamase gene from *Haemophilus* is nevertheless consistent with the ability of transposons to cause insertion mutations (12, 16). It is perhaps surprising that there is apparently one (possibly two) site for insertion of the gene in *P. aeruginosa* at *ade*. This suggests relatively limited homology between the gene and the host chromosome. Similarly, in *E. coli* the gene has apparently inserted near *trp*, close to the site reported for TnA in *E. coli* (3, 21). TnC, which specifies linked resistance to streptomycin and trimethoprim, also apparently integrates at a single site on the chromosome of *E. coli* (2).

The ability of the gene specifying TEM β-lactamase to migrate between DNA molecules is well known. This has many implications for the spread of resistance to β-lactam antibiotics to different bacterial species. Because ampicillin resistance has only recently emerged in the population of *H. influenzae*, it seems likely that this species has acquired the TEM β-lactamase by contact with members of the *Enterobacteriaceae*. The molecular properties of R plasmids specifying either ampicillin or tetracycline resistance in *Haemophilus* sp. do not, however, coincide in gross terms with those of any known plasmids found in enteric organisms (7, 8; J. R. Saunders, M. H. Richmond, L. P. Elwell, and S. Falkow, manuscript in preparation). It is possible that the TEM β-lactamase, and other resistance genes, have been deposited in *H. influenzae* by transient passage of an R factor itself incapable of survival in this species. Transposition of the resistance genes to existing replicons in *Haemophilus* has therefore probably resulted in stable acquisition of these genes but not their initial vectors. The reverse of this process, that is, transfer of the β-lactamase gene from *H. influenzae* to *E. coli* and *P. aeruginosa* without concomitant retention of transfer ability, is shown in this paper.

The TEM β-lactamase confers low levels of resistance to ampicillin in *H. influenzae* relative to other bacteria. Moreover, crypticity values for β-lactam antibiotics are extremely low in this organism (18; this paper). It is known in gram-negative bacteria that resistance to these antibiotics depends not only on the possession of a β-lactamase, but also on the penetration barrier afforded by the outer membrane (22, 24). The virtual absence of such a barrier in *H. influenzae* would reduce the effectiveness of the TEM β-lactamase in protecting the organism. Hence the relatively long delay in acquisition of ampicillin resistance could be due both to the likelihood of successful transfer after contact

with a resistant enteric organism and to the poor selective advantage conferred by the enzyme in *Haemophilus*.

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