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Development of gene probes and evolutionary relationships of the PSE-4 *bla* gene to plasmid-mediated β-lactamases of gram-negative bacteria

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Six types of plasmid-mediated carbenicillinases can be distinguished on the basis of their substrate profiles, molecular mass isoelectric values and immunological properties. As yet, no structural classification has been attempted for these enzymes at the molecular level. We have isolated the PSE-4 structural gene responsible for carbenicillinase production in *Pseudomonas aeruginosa* strain Dalgleish and studied its expression in *E. coli*. A detailed physical map of the cloned fragment and the construction of deletion mutants permitted the precise localization of the PSE-4 structural gene. Various restriction endonuclease fragments known to be flanking or internal to the PSE-4 *bla* gene were used as DNA probes and tested for homologous sequences in other β-lactamase genes. A collection of three restriction fragment probes internal or delimiting the PSE-4 structural gene were hybridized with purified plasmid DNA coding for 18 other β-lactamases. Under high stringency conditions, only the PSE-1, CARB-3 and CARB-4 genes cross-hybridized with PSE-4; while one of the probes tested hybridized solely with CARB-3. Further analysis indicated that the PSE-1, PSE-4, CARB-3 and CARB-4 *bla* genes are related and could presumably have evolved from a common progenitor.

KEYWORDS: β-lactamase, DNA probes, antibiotic resistance, carbenicillinase, gene family evolution.

INTRODUCTION

β-Lactamases (EC 3.5.2.6) are microbial enzymes responsible for hydrolysis of penicillins, cephalosporins and related β-lactam antibiotics. In gram-negative bacteria, these enzymes may be encoded either by plasmid or chromosomal genes.[1] Of the former, more than 30 biochemically distinct types have been identified.[2–4] The classification of the PSE-like enzymes has also been complicated by the transfer of β-lactam resistance without the detection of plasmids and the discovery of

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multiresistance β-lactamase transposons.[5,6] The PSE-4 β-lactamase was first detected in the Dalgleish strain of *Pseudomonas aeruginosa*[7] and is markedly active against carbenicillin.[8] Recently, it has also been shown that the PSE-4 transposons, Tn*2521* and Tn*1405*, have a preference for chromosomal insertions.[6,9] According to the Sykes and Matthew (1976) and Medeiros (1984) classification, six different types of plasmid-mediated carbenicillinases can be distinguished on the basis of their substrate profiles, molecular mass and isoelectric values. Antisera directed against the CARB-3 carbenicillinase is known to have cross-inhibitory effects on PSE-1, PSE-4 and CARB-4.[10,11] This suggested that four of the six carbenicillinases are antigenically related. However, no structural classification has been attempted at the DNA level for these enzymes.

Although the PSE-4 β-lactamase has been identified in various *P. aeruginosa* strains, this enzyme had not been previously observed in other bacterial species.[12,13] Recently, it has been shown that there is a differential expression of genes for the PSE-4 β-lactamase in *P. aeruginosa* and the enterobacteriaceae.[14] Thus, the PSE-4 β-lactamase has been identified for the first time in two non-pseudomonal strains. In addition, Williams *et al.* found PSE-4 to be the commonest β-lactamase in *P. aeruginosa*.[15] This enzyme was more prevalent than the ubiquitous TEM-1, known to be predominant in diverse enterobacteriaceae. The PSE-4 β-lactamase has also been associated with the specific serotype 0:16 of *P. aeruginosa*.[16]

Because of the implied clinical and epidemiological consequences of these observations and because of its unique properties, we initiated studies of the PSE-4 *bla* gene. In this paper, we describe the cloning of the PSE-4 structural gene and present data on its physical characterization. Using nucleic acid hybridization, we also present evidence on the evolutionary relationships of the PSE-4 β-lactamase to the other carbenicillinases.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli Strain HB101 *F⁻ ara-14 galK2 hsdS20 (hsdR hsdM) lacY1 mtl-1 proA2 recA13 rpsL20 supE44 thi xyl-5*[17] was used as standard plasmid recipient for cloning and subcloning experiments. For antibiotic susceptibility testing to streptomycin, we transformed the recombinant plasmids in *E. coli* C600 *F⁻ lacY leu thi thr*[18] and tests were done as described previously.[19] The bacterial plasmids coding for various β-lactamase types are given in Table 1.

Genetic abbreviations used: *aadA*, aminoglycoside 3″-adenyltransferase; *bla*, β-lactamase; *cat*, chloramphenicol acetyltransferase; *Hg*, mercuric ion resistance; *sul*, sulphonamide resistance.

Preparation of plasmid DNA

Plasmid DNA was prepared using the cleared-lysate method[27] followed by cesium chloride-ethidium bromide gradient ultracentrifugation.[28] Recombinant plasmids were constructed and analysed as described by Levesque *et al.*, 1987.[19]

Table 1. Description of plasmids used in this study

Plasmid	β-Lactamase	pI	Relevant phenotype[a]	Reference
pBR322	TEM-1	5·4	Ap Tc	20
pMON62	TEM-1	5·4	Ap Cm	19
pMK20/RP4	TEM-2	5·6	Ap Km	19
pMON301	OXA-1	7·4	Ap Cm	19
pMON21	OXA-2	7·7	Ap Cm	21
pCR1::Tn*1411*	OXA-3	7·1	Ap Cm Gm Km Sm Su Tm	19
pMON102	OXA-4	7·5	Ap Cm	19
pMON53	OXA-5	7·62	Ap Cm Sm Su	19
pMON31	SHV-1	7·6	Ap Cm	Mercier *et al.*, submitted
pMON810	PSE-1	5·7	Ap Cm	19
pMON230	PSE-2	6·1	Ap Cm	19
pMON225	PSE-3	6·9	Ap Cm	19
pMON41	CARB-3	5.75	Ap Cm Sm	19
pMON1025	CARB-4	4·3	Ap Cm Gm Km Su Tm	19
pMON80	LCR-1	6·5	Ap Cm Kc	19
pMON510	AER-1	5·9	Ap Cm	19
pMON401	ROB-1	8·1	Ap Cm	19
pNU81	CEP-1(*ampC*)	9·8	Ap Km	22
R388	None		Su Tp	23
pCR1	None		Km	24
pACYC184	None		Cm Tc	25
pMK20	None		Km	24
pUZ8	None		Hg Km Tc	26
pMON700	PSE-4	5·3	Ap Cm Sm Su	This work
pMON701	PSE-4	5·3	Ap Cm Sm Su	This work
pMON703	PSE-4	5·3	Ap Cm Sm	This work
pMON705	PSE-4	5·3	Ap Cm Sm	This work
pMON707	PSE-4	5·3	Ap Cm Sm	This work
pMON708	——		Cm Sm	This work
pMON709	PSE-4	5·3	Ap Cm	This work

[a]Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Hg, mercuric chloride; Km, kanamycin; Sm, streptomycin; Su, sulphonamide; Tc, tetracycline; Tm, tobramycin; Tp, trimethoprim.

Preparation of DNA probes and hybridization techniques

Restricted DNA fragments were separated by agarose or polyacrylamide electrophoresis gels and electroeluted.[21,29] Approximately 0·5 μg of each DNA fragment used as probe was labelled with 50 μCi of ^{32}P-dCTP by nick translation.[30]

Purified plasmid DNAs[31] (0·5 μg) were spotted on nitrocellulose filters. After prehybridization, the filters were hybridized under medium to high stringency conditions at 42°C for 18 h with 40% formamide in 6 × SSC (1 × SSC is 0·15 M NaCl, 0·05 M sodium citrate, pH 7·0) followed by washes in 0·1 × SSC at 42, 52 or 65°C.[21] Autoradiography was done according to Maniatis *et al.*, 1982.[29] Exposure time varied from 18 h to 96 h.

RESULTS

Isolation of the PSE-4 structural gene

The original PSE-4 β-lactamase gene from *P. aeruginosa* strain Dalgleish has been transposed from the prototype plasmid Ps. Dal into pUZ8 and then into pUB5573 so as to facilitate studies in *E. coli*[6]. The resulting plasmid, pUB5573::Tn*1405*, was purified, digested with *Bam*HI and the fragments obtained were ligated with pACYC184 digested with the same enzyme. The recombinant molecules obtained were introduced into *E. coli* HB101 by transformation and selected for chloramphenicol and ampicillin resistance and tetracycline susceptibility. On the basis of this phenotype, a series of recombinant plasmids were screened by restriction enzyme analysis with *Bam*HI and *Hin*dIII so as to select inserts in opposite orientations. Using this approach, two clones were selected, pMON700 and pMON701, and confirmed to produce the prototype PSE-4 β-lactamase by isoelectric focusing (data not shown). Further analysis demonstrated that the plasmids selected, pMON700 and pMON 701, also coded for resistance to streptomycin and sulphonamide in addition to ampicillin resistance (Table 1).

Physical mapping and structural features of the PSE-4 *bla* gene

Using a series of single and double digests, the pMON700 and pMON701 recombinant plasmids were mapped with *Ava*I, *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII and *Pvu*II (Fig. 1). Both plasmids were confirmed to have the same 4·4 Kb *Bam*HI restriction fragment also found in Tn*1405*, but in opposite orientations. In order to localize the PSE-4 structural gene, a series of deletion plasmids were constructed. By producing the *Hin*dIII deletion plasmid pMON703, the sulphonamide resistance gene was localized in the *Hin*dIII-*Bam*HI region of pMON700. Further deletions of *Ava*I and *Bam*HI-*Bgl*II fragments localized the *bla* gene in the *Bgl*II region of pMON707. The resulting plasmids, pMON703, pMON705 and pMON707 are shown in Fig. 1. Additional fine restriction mapping using *Bgl*I, *Bst*EII, *Hin*cII and *Xba*I in pMON707 permitted to localize the streptomycin resistance gene in the *Bst*EII region. A *Bst*EII-*Hin*dIII digest, Klenow fill-in ligation construction gave pMON709 with an insert of 1·27 kb. The resulting *E. coli* transformants were confirmed to produce the prototype PSE-4 β-lactamase by isoelectric focusing.

Hybridization of PSE-4 with other *bla* genes

Four nucleic acid probes known to be internal to the PSE-4 *bla* gene or flanking it in the Tn*1405* transposon were tested for homologous DNA sequences with other *bla* plasmids (Fig. 2). We first decided to test DNA probes that would presumably be flanking the PSE-4 structural gene. The first probe tested was a 430 bp *Bst*EII-*Bgl*I fragment known to contain the major part of the *aadA* gene and part of the PSE-4 gene. This probe hybridized strongly with the PSE-4-harbouring plasmid but also to plasmid DNA encoding OXA-2, PSE-2 and CARB-3 [Fig. 3(a)]. Also, detectable but less intense cross-hybridization could also be detected with TEM-1 and AER-1

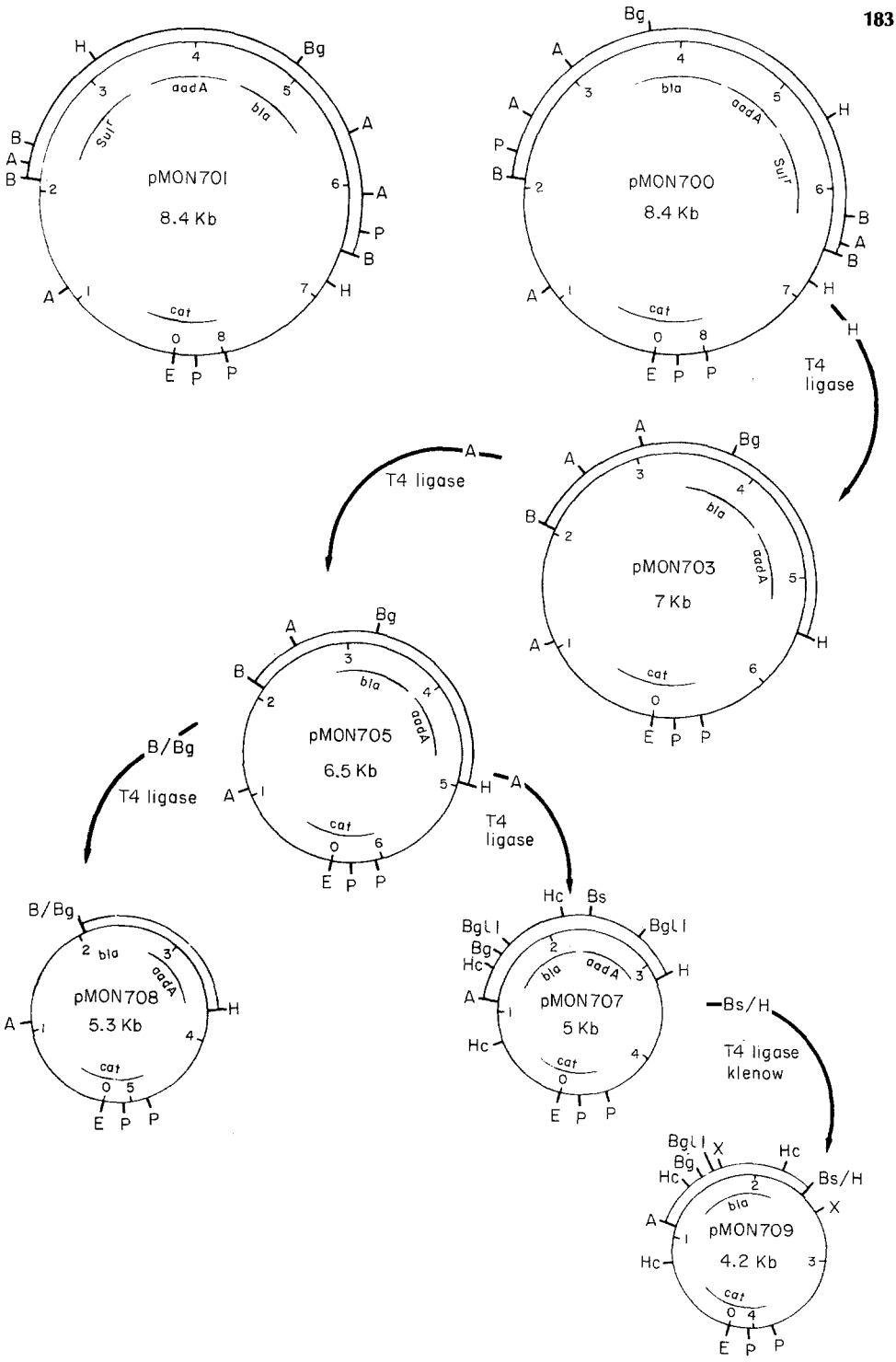

Fig. 1. Isolation of the PSE-4 *bla* gene. Plasmids pMON700 and pMON701 were constructed by cloning a 4·4 Kb *Bam*HI DNA fragment from pUB5573::Tn*1405* (Levesque & Jacoby, 1988) into the *Bam*HI site of pACYC184 in both orientations. A series of subsequent deletions gave the pMON709 construction and localized the PSE-4 structural gene in the *Bgl*II-*Bst*EII region. Abbreviations are: *bla*, beta-lactamase; *Cat*, chloramphenicol acetyl transferase; Sm, streptomycin; sul, sulphonamide. Restriction enzyme sites shown are as follows: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; and X, *Xba*I.

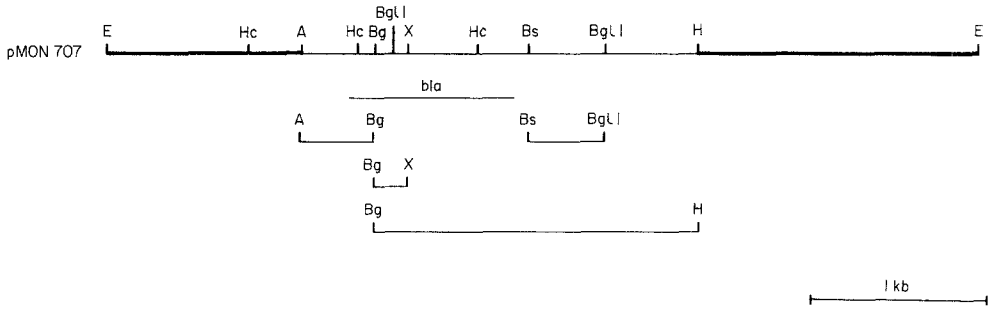

Fig. 2. Physical map of the pMON707 recombinant plasmid showing the restriction endonuclease fragments used as probes. The thin line represents the Tn*1405* 4·4 Kb *Bam*HI DNA fragment insert and the thick line the pACYC184 vector DNA. Abbreviations are: A, *Ava*I; *bla*, β-lactamase; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; Kb, kilobase; X, *Xba*I.

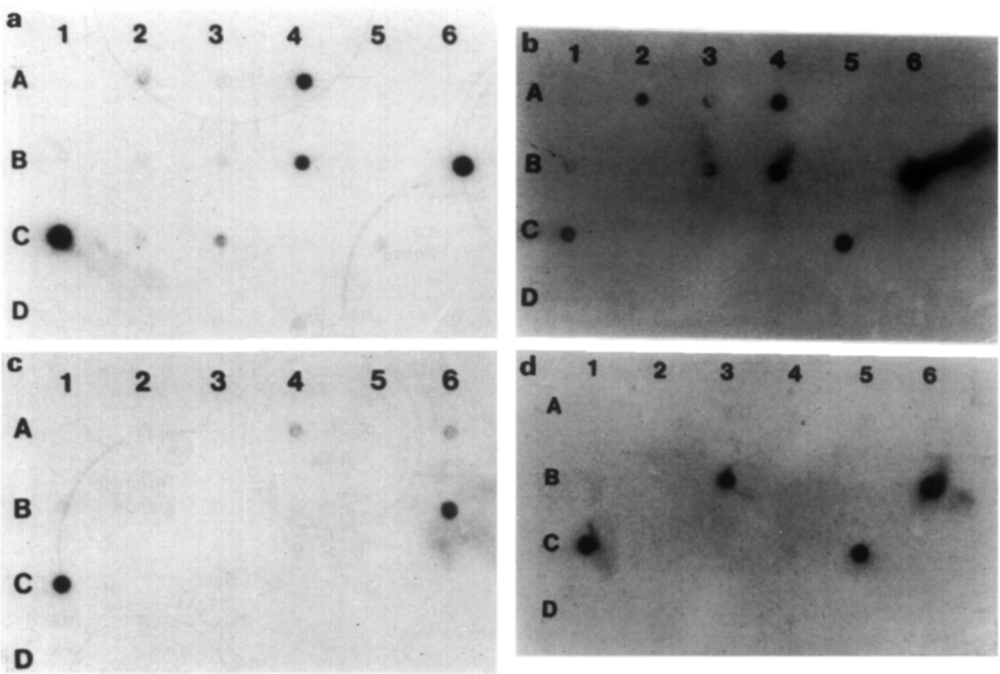

Fig. 3. Dot-blot hybridization using equivalent amounts (500 ng) of purified recombinant plasmid DNA carrying cloned *bla* genes. These results were obtained using as probes the 430 bp *Bst*EII-*Bgl*I fragment (a); the 1·7 Kb *Bgl*II-*Hin*dIII (b); the 450 bp *Ava*I-*Bgl*II fragment (c) and the 170 bp *Bgl*I-*Xba*I fragment (d) from pMON707. Positions in the figure are as follows: A1, pBR322 (TEM-1); A2, pMON62 (TEM-1); A3, pMON301 (OXA-1); A4, pMON21 (OXA-2); A5, pCR1::Tn*1411* (OXA-3); A6, pMON102 (OXA-4); B1, pMON53 (OXA-5); B2, pMON31 (SHV-1); B3, pMON810 (PSE-1); B4, pMON230 (PSE-2); B5, pMON225 (PSE-3); B6, pMON705 (PSE-4); C1, pMON41 (CARB-3); C2, pMON401 (ROB-1); C3, pMON510 (AER-1); C4, pMON80 (LCR-1); C5, pMON1025 (CARB-4); C6, pNU81 (CEP-1); D1, pMK20/RP4 (TEM-2); D2, R388 (None); D3, pCR1 (None); D4, pACYC184 (None); D5, pMK20 (None); D6, pUZ8 (None). Hybridizations were performed under medium to high stringency conditions.

plasmid DNAs. This was expected since it is well known that in many cases *bla* genes are generally flanked by the *aadA* gene.[6] The second probe tested was a 1·7 kb *Bgl*II-*Hin*dIII fragment known to contain most of the PSE-4 *bla* gene and also the flanking *aadA* gene (Fig. 2). Strong cross-hybridization signals were detected with plasmid DNA coding for OXA-2, PSE-2 and CARB-4 [Fig. 3(b)]. Lower but significant signals were also detected with TEM-1 and CARB-3 plasmid DNA. Again, it was evident that probes specific for *bla* genes have to be within the structural gene and strictly defined. The second series of PSE-4 probes tested were assumed to be mostly internal to the structural *bla* gene; as would be expected from the construction of deleted plasmids shown in Fig. 1. In addition to self-hybridization, the 450 bp *Ava*I-*Bgl*II probe shared homologous sequences with CARB-3 [Fig. 3(c)]. A weak but non-significant cross-hybridization signal which could easily be discriminated was seen with OXA-2 and OXA-4 bearing plasmids. To better define the DNA homologies detected, we prepared a specific PSE-4 *bla* probe using the 170 bp *Bgl*II-*Xba*I fragment (Fig. 2). Since the 450 bp *Ava*I-*Bgl*II fragment is essential for β-lactamase expression, we would expect this 170 bp fragment to be completely within the PSE-4 structural gene. This probe was tested and cross-hybridization was detected with PSE-1, CARB-3 and CARB-4 [Fig. 3(d)]. Finally, similar results were obtained when using the 720 bp *Bgl*I-*Bst*EII fragment known to delimit the PSE-4 *bla* gene (data not shown). Thus, it is apparent that on an evolutionary basis, the PSE-4, CARB-3, CARB-4 and PSE-1 structural genes are related and could have evolved from a common ancestor. It is also evident that for the development of specific *bla* gene probes restriction endonuclease fragments selected have to be completely within their respective structural gene and systematically evaluated against all other known *bla* genes.

DISCUSSION

Plasmid-mediated β-lactamases are remarkable enzymes which have a unique capability for diversification. For example, it has been shown in a number of instances, that minor changes in amino acid structure of these enzymes may result in fully functional proteins with no significant modifications in substrate profiles.[32] In contrast, other minor amino acid changes may yield mutant TEM-like β-lactamases capable of hydrolysing the novel β-lactam compounds.[33]

By molecular cloning techniques, we have isolated 27 of the more than 30 biochemically distinct *bla* genes and are using them as a model to study: (a) *bla* gene evolution and polymorphisms; and (b) for the development of *bla* gene probes.[19,21]

Although DNA probes have already been developed for the TEM-like and OXA-like *bla* genes, there were no specific PSE probes available.[19,21,34–36] Because of the unique and interesting properties of the PSE-4 β-lactamase, we present data on the initial steps of its molecular characterization. Recombinant plasmids were constructed for convenient isolation of restriction fragments flanking or internal to the PSE-4 structural gene. Preliminary experiments showed that the 1·7 kb *Bgl*II-*Hin*dIII and the 430 bp *Bst*EII-*Bgl*I fragment cross-hybridized with OXA-2, PSE-2, CARB-3, CARB-4 and TEM-1 bearing plasmids; a result due in part to sequences outside the PSE-4 *bla* gene. These plasmid-mediated β-lactamases are part of transposons Tn*2410*, Tn*1404*, Tn*1408*, Tn*1413* and Tn*Aβ*, which are complex units carrying

additional similar resistance genes to spectinomycin, streptomycin and sulphonamide.[6] Cross-hybridization observed could presumably be due to the presence of these genes. In addition, an extraordinary 1·2 kb *Bam*HI-*Ava*I-*Ava*I fragment upstream of the *aadA* gene found in Tn*21* is identical (in terms of restriction fragments and DNA homology) to corresponding regions adjacent to almost all *bla* genes including PSE-4. This is not a locus encoding known antibiotic resistances but is part of a cryptic region encoding the *tnpI* gene and known to be implicated in the genesis of multiresistance transposons by an integrase-like mechanism (R. C. Levesque, J. Mercier, A. Bergeron & M. Lafond, *Abstracts of the Annual Meeting of the American Society of Microbiology*, 1987, H102, p. 156). We have also found this region in a variety of resistance plasmids and its nucleotide sequence as well as its integrase function has been elucidated in Tn*21* and in R46 (Mercier *et al.*, submitted for publication).[37,38]

On a routine basis in a clinical setting and for epidemiological studies, specific identification of β-lactamases such as TEM-1(pI:5·4), TEM-2(pI:5·6), PSE-1(pI:5·7), PSE-4(pI:5·3), CARB-3(pI:5·75), CARB-4(pI:4·9) and AER-1(pI:5·9) by pI is a difficult task. The problem is almost impossible when one is dealing with hundreds of bacterial isolates. The experimental data presented here was initially started for the construction of a specific PSE-4 *bla* probe so as to facilitate its differentiation from other β-lactamases with similar isoelectric points. To date it is apparent that when hybridization experiments are done at high stringency conditions, one of the PSE-4 probes that we tested can discriminate between all plasmid-mediated β-lactamases; except for CARB-3. In contrast, the two other PSE-4 probes tested known to be internal or delimited by the structural gene confirmed that PSE-1, PSE-4, CARB-3 and CARB-4 are related and may be variants of the same *bla* gene having a common ancestor. Nucleotide sequencing as well as oligonucleotide probes will be necessary for elucidating their evolutionary relationships and for their specific identification by nucleic acid hybridization.

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REFERENCES

1. Sykes, R. B. & Matthew, M. (1976). The β-lactamases of gram-negative bacteria. *Journal of Antimicrobial Chemotherapy* **2**, 115–57.
2. Medeiros, A. A. (1984). β-lactamases. *British Medical Bulletin* **40**, 18–27.
3. Medeiros, A. A., Cohenford, M. & Jacoby, G. A. (1985). Five novel plasmid-determined β-lactamases. *Antimicrobial Agents and Chemotherapy* **27**, 715–9.
4. Bush, K. (1988). Recent development in β-lactamase research and their implications for the future. *Reviews of Infectious Disease* **10**, 681–90.
5. Ingram, L., Sykes, R. B., Grinsted, J., Saunders, J. R. & Richmond, M. H. (1972). A transmissible resistant element from a strain of *Pseudomonas aeruginosa* containing no detectable extrachromosomal DNA. *Journal of General Microbiology* **72**, 269–79.

6. Levesque, R. C. & Jacoby, G. A. (1988). Isolation and interrelationships of multiresistance β-lactamase transposons. *Plasmid* **19**, 21–9.
7. Newsom, S. W. B. (1969). Carbenicillin-resistant Pseudomonas. *Lancet* **2**, 1140.
8. Newsom, S. W. B., Sykes, R. B. & Richmond, M. H. (1970). Detection of a β-lactamase markedly active against carbenicillin in a strain of *Pseudomonas aeruginosa*. *Journal of Bacteriology* **101**, 1079–80.
9. Sinclair, M. I. & Holloway, B. W. (1982). A chromosomally located transposon in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **151**, 569–79.
10. Labia, R., Guionie, M., Barthélémy, M. & Phillipon, A. (1981). Properties of three carbenicillin-hydrolysing β-lactamases (CARB) from *Pseudomonas aeruginosa*: identification of a new enzyme. *Journal of Antimicrobial Chemotherapy* **7**, 49–56.
11. Philippon, A., Paul, G., Thabaut, A. & Jacoby, G. A. (1986). Properties of a novel carbenicillin-hydrolysing β-lactamase (CARB-4) specified by an IncP2 plasmid from *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **29**, 519–20.
12. Matthew, M. (1979). Plasmid-mediated β-lactamases of gram-negative bacteria: properties and distribution. *Journal of Antimicrobial Chemotherapy* **5**, 349–58.
13. Foster, T. J. (1983). Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiology Reviews* **47**, 361–409.
14. Reid, A. J., Simpson, I. A., Harper, P. A. & Amyes, B. G. (1988). The differential expression of genes for the PSE-4 β-lactamase in *Pseudomonas aeruginosa* and the enterobacteriaceae. *Journal of Antimicrobial Chemotherapy* **21**, 525–33.
15. Williams, R. J., Livermore, D. M., Lindridge, M. A., Said, A. A. & Williams, J. D. (1984). Mechanisms of β-lactam resistance in British isolates of *Pseudomonas aeruginosa*. *Journal of Medical Microbiology* **17**, 283–93.
16. Livermore, D. M., Pitt, T. L., Jones, C. S., Crees-Morris, J. A. & Williams, R. J. (1985). PSE-4 β-lactamase: a serotype specific enzyme in *Pseudomonas aeruginosa*. *Journal of Medical Microbiology* **19**, 45–53.
17. Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *Journal of Molecular Biology* **41**, 459–72.
18. Bachmann, B. J. (1972). Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriology Reviews* **36**, 525–57.
19. Levesque, R. C., Medeiros, A. A. & Jacoby, G. A. (1987). Molecular cloning and DNA homology of plasmid-mediated β-lactamases. *Molecular and General Genetics* **206**, 252–8.
20. Sutcliffe, J. G. (1978). Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. *Proceedings of the National Academy of Sciences USA* **75**, 3737–41.
21. Boissinot, M., Mercier, J. & Levesque, R. C. (1987). Development of natural and synthetic DNA probes for OXA-2 and TEM-1 β-lactamases. *Antimicrobial Agents and Chemotherapy* **31**, 728–34.
22. Jaurin, B., Grundstrom, T. & Normark, S. (1982). Sequence elements determining *ampC* promoter strength in *E. coli*. *EMBO Journal* **1**, 875–81.
23. Ward, J. M. & Grinsted, J. (1982). Physical and genetic analysis of the IncW group plasmids R388, Sa and R7K. *Plasmid* **7**, 239–50.
24. Kahn, M., Kolter, R., Thomas, C., Figurski, D., Keyer, R., Remaut, E. & Helinsky, D. R. (1979). Plasmid cloning vehicles derived from plasmids ColE, F, R6K and RK2. *Methods in Enzymology* **68**, 268–80.
25. Chang, A. C. Y. & Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *Journal of Bacteriology* **134**, 1141–56.
26. Leemans, J., Inzé, D., Villaroel, R., Engler, G., Hernalsteens, J. P., De Block, M. & Van Montagu, M. (1981). Plasmid mobilization as a tool for in vivo genetic engineering. In *Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids* (Levy, S. D., Clowes, R. C. & Koening, E. L., eds), pp. 401–9. New York: Plenum Press.
27. Guerry, P., Leblanc, D. J. & Falkow, S. (1973). A general method for the isolation of plasmid deoxyribonucleic acid. *Journal of Bacteriology* **16**, 1064–6.
28. Radloff, R., Bauer, W. & Vinograd, J. (1967). A dye buoyant-density method for the detection and isolation of close circular duplex DNA: the close circular DNA in HeLa cells. *Proceedings of the National Academy of Sciences USA* **57**, 1514–21.
29. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). In *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.

30. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977). Labelled deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *Journal of Molecular Biology* **113**, 237–51.
31. Kafatos, F. C., Jones, C. W. & Efstradiadis, A. (1979). Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. *Nucleic Acids Research* **7**, 1541–52.
32. Schultz, S. C. & Richards, J. H. (1986). Site-saturation studies of β-lactamase: Production and characterization of mutant β-lactamases with all possible amino acid substitutions at residue 71. *Proceedings of the National Academy of Sciences USA* **83**, 1588–92.
33. Sougakoff, W., Goussard, S., Gerbaud, G. & Courvalin, P. (1988). Plasmid-mediated resistance to third-generation cephalosporins caused by point mutations in TEM-type penicillinase genes. *Review of Infectious Diseases* **10**, 879–84.
34. Cooksey, R. C., Clark, N. C. & Thornsberry, C. (1985). A gene probe for TEM type β-lactamases. *Antimicrobial Agents and Chemotherapy* **28**, 154–6.
35. Ouellette, M. & Roy, P. H. (1986). Analysis by using DNA probes of the OXA-1 β-lactamase gene and its transposon. *Antimicrobial Agents and Chemotherapy* **30**, 46–51.
36. Huovinen, S., Huovinen, P. & Jacoby, G. A. (1988). Reliability of biotinylated DNA probes in colony hybridization: evaluation of an improved colony lysis method for detection of TEM-1 β-lactamase. *Molecular and Cellular Probes* **2**, 83–5.
37. Hall, R. & Vockler, C. (1987). The region of the IncN plasmid R46 coding for resistance to β-lactam antibiotics, streptomycin/spectinomycin and sulphonamides is closely related to antibiotic segments found in IncW plasmids and in Tn*21* transposons. *Nucleic Acids Research* **15**, 7491–501.
38. Martinez, E. & de la Cruz, F. (1988). Transposon Tn*21* encodes a *RecA* independent site-specific integration system. *Molecular and General Genetics* **211**, 320–5.