PSE-4 BETA LACTAMASE: A SEROTYPE-SPECIFIC ENZYME IN *PSEUDOMONAS AERUGINOSA*

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SUMMARY. PSE-4 enzyme is the most common plasmidic β lactamase in *Pseudomonas aeruginosa* but its production is invariably non-transferable by conjugation. Of 20 PSE-4⁺ isolates from 10 separate sources, 16 were serotype O:16 and two belonged to the related O:2(b) serotype. One of the two remaining organisms was not O-typable and the other was agglutinated by several unrelated antisera. Examination of additional O:16 strains confirmed the unusual frequency of PSE-4 enzyme in this serotype. None of the PSE-4⁺ strains was able to transfer carbenicillin resistance in mating experiments and none contained extrachromosomal DNA. Two explanations of the relationship between enzyme production and O antigenicity are proposed. PSE-4 production may be transmissible, perhaps by transduction, between strains of O:16 or related serotypes, or PSE-4⁺ *P. aeruginosa* may represent a disseminated subtype. A third hypothesis, that the PSE-4 coding element carried serotype determinants, was discounted. PSE-4⁺ and PSE-4⁻ *P. aeruginosa* strains of O:16 and related serotypes were found to represent a definite cluster by their phage-susceptibility pattern and pyocin type (type 1). The only characters linked to PSE-4 production were resistance to spectinomycin, streptomycin and sulphonamide and the genes responsible for these characters seemed to occur on the PSE-4 coding element.

INTRODUCTION

Various plasmid-mediated β lactamases, may cause penicillin and cephalosporin resistance in gram-negative bacteria (Matthew, 1979; Foster, 1983). Most of these enzymes have now been observed in *Pseudomonas aeruginosa* (Jacoby, Sutton and Medeiros, 1980) but their frequency in this species remains low. In a recent multicentre survey we detected β lactamases considered by Foster (1983) to be plasmidic in only 2% of 1866 *P. aeruginosa* isolates (Williams *et al.*, 1984). In contrast, Whitaker, Hajipieris and Williams (1983) detected these enzymes in 43% of 1000 gram-negative rods

isolated from patients at The London Hospital during 1982. The frequency of various enzyme types also varied between *P. aeruginosa* and other gram-negative rods; in *P. aeruginosa* we found that PSE-4 (Dalglish enzyme) (Newsom, Sykes and Richmond, 1970) was the most common (Williams *et al.*, 1984) whereas TEM-1 is by far the most frequent amongst gram-negative rods as a whole (Matthew, 1979; Whitaker *et al.*, 1983). Other groups have reported PSE-4 production by *P. aeruginosa* strains from Great Britain (King *et al.*, 1980), France (Labia *et al.*, 1977; Jouvenot, Bonin and Michel-Briand, 1983), the Netherlands (Stobberingh, Houben and van Boven, 1982) and Australia (Sinclair and Holloway, 1982), confirming that this enzyme is widely scattered in the species. PSE-4 is, however, unknown outside *P. aeruginosa* (Matthew, 1979; Foster, 1983) and its production is invariably non-transmissible by conjugation even within the species (Jacoby and Matthew, 1979). Recent studies on Australian PSE-4⁺ isolates have indicated that enzyme production is encoded by a transfer-deficient chromosomally-integrating transposon (Sinclair and Holloway, 1982).

The wide distribution of PSE-4 β lactamase thus presents a paradox and this has been investigated in the present study. Enzyme production has been shown to be almost restricted to a single serogroup and a close degree of similarity between strains has been demonstrated within this group.

MATERIALS AND METHODS

Strains of P. aeruginosa. Eighteen isolates that produced PSE-4 co-focusing β lactamases were from a national survey of antibiotic resistance in *P. aeruginosa* (Williams *et al.*, 1984). Two PSE-4⁺ isolates were from an earlier study (Livermore, Williams and Williams, 1981). *P. aeruginosa* Dalglish (Newsom *et al.*, 1970) was used as a PSE-4-producing reference strain. Forty-one strains which reacted with O sera against two or more of the antigenically related group-II serotypes O:2(a), O:2(b), O:5 and O:16 and 30 further strains of serotype O:16 were received by the Division of Hospital Infection, Central Public Health Laboratory, Colindale, during 1981–1982. Strains of other serotypes were from our culture collection. *P. aeruginosa* PU21 *ilv leu rif str* was described by Jacoby (1974) and plasmid pUZ8, contained in *Escherichia coli* K12 J53-2 *pro met nal*, was described by Hedges and Matthew (1979).

Typing of cultures. Cultures were serotyped according to the International Antigenic Typing Scheme described by Liu *et al.* (1983) by the methods of Pitt (1981). Antisera against the type strains of serotypes O:2(b) and O:5(d) of Veron (1961) were used for the sub-division of serotypes O:2 and O:5. Phage typing was performed as described by Asheshov (1974) and pyocin typing as described by Govan and Gillies (1969).

Minimum inhibitory concentrations (MICs) were determined by an agar dilution technique with doubling dilutions of antibiotics in Diagnostic Sensitivity Test (DST) agar (Oxoid CM261). Inocula contained *c.* 10⁴ cells from overnight nutrient-broth culture. Results were read after incubation for 18 h at 37°C and MICs were defined as the lowest antibiotic concentration at which there was no visible growth.

Examination of β -lactamase production. Carbenicillin-resistant organisms (MIC >128 mg/L) were examined for constitutive β -lactamase expression by the EDTA-nitrocefin technique (Williams *et al.*, 1984). Extracts containing β lactamase were prepared from organisms that gave a positive reaction by the 'washed slope' method of Livermore, Maskell and Williams (1984) and were subjected to isoelectric focusing by the method of Matthew *et al.* (1975). Enzyme identification was by comparison with standard TEM-1 and 2, OXA-1, 2 and 3, PSE-1, 2, 3 and 4, SHV-1 and HMS-1 β lactamases. Larger amounts of β lactamases for enzyme activity studies were prepared from logarithmic phase nutrient-broth cultures by the method of Williams *et al.* (1984) with the modification that enzyme was liberated by two freeze-thaw cycles instead of by sonication. Hydrolytic activity was examined by UV spectrophotometric assay (O'Callaghan,

Muggleton and Ross, 1969; Waley, 1974) with substrates at 1 mM concentration in 0.1 M phosphate buffer pH 7.0. Substrates and absorption wavelengths were as follows: benzyl penicillin (Glaxo Group Research), 235 nm; carbenicillin (Beecham Research Laboratories), 235 nm; oxacillin (Sigma Chemical Co.), 263 nm; and cephaloridine (Glaxo Group Research), 295 nm.

Genetic studies on PSE-4 production. Conjugative transfer of carbenicillin resistance from PSE-4⁺ isolates to *P. aeruginosa* strain PU21 was initially attempted by mating the organisms in nutrient broth (Livermore and Williams, 1982). Modifications of this method were also attempted whereby donor and recipient were mixed either on nutrient agar plates or on 0.45- μ m pore size membrane filters (Millipore Corporation). Transconjugants were selected on DST agar containing rifampicin (Gruppo Lepetit SA, Milan) 100 mg/L and carbenicillin 250 mg/L.

Transfer of the PSE-4 coding element from *P. aeruginosa* Dalglish was also attempted by a recombinant plasmid technique. This organism was grown overnight in 10 ml of nutrient broth and the cells were harvested at 5000 *g* and resuspended in 0.5 ml of a 50 $\times$ concentrated saline suspension of a 6-h nutrient-broth culture of *E. coli* J53-2 (pUZ8). The mixture (100 μ l) was spread across a DST plate and incubated for 24 h at 37°C. Cells were then washed into saline 0.9% w/v and transferred to DST agar containing tetracycline (Lederle) 50 mg/L and cefoxitin (Merck, Sharpe and Dohme) 100 mg/L. *P. aeruginosa* Dalglish transconjugants with the pUZ8 plasmid were picked off after incubation for 2 days at 37°C and confirmed as oxidase positive and kanamycin resistant (MIC > 256 mg/L). One of these transconjugants was then mated with *P. aeruginosa* strain PU21 by growing both organisms overnight in 10-ml volumes of nutrient broth. The cells were harvested at 5000 *g* and the pellets mixed in 0.5 ml of saline, 0.1 ml of which was spread on a DST agar plate. After overnight incubation the cells were washed into saline and transferred to DST agar containing carbenicillin 250 mg/L, kanamycin 200 mg/L and rifampicin 100 mg/L. Transconjugants, designated PU21 (pUZ8 : : PSE-4) were confirmed as producers of PSE-4 β lactamase by isoelectric focusing and as auxotrophs by their inability to grow on mineral salts agar containing D-glucose 0.5% w/v as sole carbon and energy source. *P. aeruginosa* strain PU21 (pUZ8 : : PSE-4) was mated with prototrophic *P. aeruginosa* isolates of various serotypes by the same method used for crosses between *P. aeruginosa* Dalglish (pUZ8) and strain PU21 with the modification that transconjugant selection was on mineral salts agar containing D-glucose 0.5% w/v and carbenicillin 250 mg/L.

Organisms were examined for plasmid DNA by the method of Kado and Liu (1981). Plasmid electrophoresis was on 0.7% agarose vertical slab gels with a 'Protean' apparatus (Bio-Rad Laboratories, Watford). The gel thickness was 3 mm and a constant voltage of 160 V was employed. Electrophoresis buffer was 40 mM Tris-acetate, pH 7.8, containing 2 mM EDTA. Plasmids were visualised by staining with aqueous ethidium bromide 0.5 mg/L for 30 min and photographed, with UV illumination, with a Polaroid CU-5 Land camera with type 655 or 667 film. Plasmid DNA for restriction endonuclease analysis was extracted by a scaled up version of the method of Kado and Liu (1981) that used 50 ml of culture. Restriction cleavage was performed with enzymes *Eco*RI and *Hind*III (Sigma Chemical Co., St Louis, MO); the methods were those recommended by the manufacturers. Fragments were electrophoresed in 0.8% agarose gels using a horizontal apparatus (Bio-Rad DNA 'Sub-Cell'). Buffers and visualisation methods were as for plasmid electrophoresis.

RESULTS

Serotyping of PSE-4⁺ P. aeruginosa

Twenty *P. aeruginosa* isolates that produced β lactamases which aligned with PSE-4 enzyme from *P. aeruginosa* on isoelectric focusing gels were examined initially. These organisms were from our survey and from earlier collections. Sixteen organisms, from eight separate sources, reacted with antiserum to type O:16 and two, from separate sources, reacted with antiserum to type O:2(b). These serotypes are closely related. Of the remaining two organisms one was not agglutinated by any antiserum

and was termed not typable and the other was agglutinated by antisera to types O:3, O:9, O:10 and O:13 and was termed polyagglutinable. *P. aeruginosa* Dalglish was serotype O:16.

Examination of O:16 and other group-II P. aeruginosa strains for carbenicillin resistance and PSE-4 production

Examination of serotype O:16 and other group-II *P. aeruginosa* strains received by the Division of Hospital Infection, Central Public Health Laboratory, Colindale revealed that 12 out of 30 serotype O:16 strains were resistant to carbenicillin 128 mg/L and seven of these produced β lactamases indistinguishable from PSE-4 by isoelectric focusing. Seven of 41 other group-II isolates were carbenicillin resistant and one produced a β lactamase of identical pI (c. 5.3) to the PSE-4 enzyme.

Comparison of PSE-4⁺ and PSE-4⁻ strains of serotype O:16 and other group-II serotypes of P. aeruginosa

The initial studies described above yielded 20 epidemiologically distinct strains of *P. aeruginosa* that produced β lactamases that aligned with PSE-4 in electrofocusing experiments (table I). Hydrolysis assays confirmed that these enzymes had similar activity to the PSE-4 enzyme from *P. aeruginosa* Dalglish (table I). None of the organisms was able to transfer carbenicillin resistance to strain PU21 in simple broth and plate mating experiments and none contained extrachromosomal DNA.

These strains were compared with 20 distinct carbenicillin-susceptible PSE-4⁻ strains, 14 of serotype O:16 and six of other group-II serotypes. All the PSE-4⁺ isolates were of pyocin type 1, as were 19 out of 20 of the PSE-4⁻ strains, the exception being a strain of serotype O:16 and pyocin type 10. PSE-4⁺ and PSE-4⁻ isolates were generally susceptible to phages 44, 68, 109 and 1214, but were usually resistant to the other phages tested. All the PSE-4⁺ strains were highly resistant to spectinomycin (MIC > 2048 mg/L), streptomycin (MIC 256-512 mg/L) and sulphamethoxazole (MIC > 1024 mg/L). PSE-4⁻ strains were much less resistant to these antibiotics.

Transfer of the determinant for PSE-4 enzyme production from P. aeruginosa Dalglish by recombinant plasmid formation

A PSE-4 enzyme-producing transconjugant, designated PU21(pUZ8::PSE-4), was obtained. Agarose gel electrophoresis of 10 separate clones indicated that this organism had acquired a single plasmid of mol. wt 38.8×10^6 in contrast to the value of 34×10^6 for plasmid pUZ8. These results indicated that a recombinant plasmid had been formed between pUZ8 and the PSE-4 coding element and that the latter had a mol. wt of 4.8×10^6 (7.2 Kb pairs). Extrachromosomal DNA was not detected in *P. aeruginosa* Dalglish and this suggested that the PSE-4 coding element was incorporated into the chromosome of this organism. Analysis of fragments obtained by endonuclease treatment did not indicate an additional site for *EcoRI* in the (pUZ8::PSE-4) plasmid compared with pUZ8 alone, but two extra sites for *HindIII* were detected.

Sero- and phage-typing results for *P. aeruginosa* Dalglish, *P. aeruginosa* Dalglish

TABLE I

Sources, serotypes and β -lactamase activities of epidemiologically distinct PSE-4⁺ strains

Strain	Source	Serotype	Relative hydrolysis rate of			
			Pen G	Carb	Ceph	Oxa
Dalgleish	Newsom <i>et al.</i> (1970)	O:16	100	167	11.1	9.1
R25	London Hospital	O:16	100	138	9.4	8.1
R57	Birmingham	O:16	100	146	10.1	8.4
M145	Irvine	O:16	100	122	10.8	8.6
M202	Bangor (Wales)	NT	100	147	9.8	8.7
M423	Birmingham	O:16	100	133	9.5	9.3
M817	Guys, London	O:16	100	127	13.3	9.1
M1235	Liverpool	O:16	100	165	12.5	10.6
M1767	St Thomas's, London	O:2(b)	100	110	10.0	7.7
M2138	York	O:16	100	148	10.9	8.9
M2207	Newcastle upon Tyne	O:16	100	114	8.3	6.5
M2225	Newcastle upon Tyne	PA	100	122	9.9	10.6
C4218	Edgware	O:16	100	136	10.2	8.4
C4918	Southampton	O:16	100	151	10.3	8.5
C5156	Kent and Sussex	O:16	100	136	9.9	7.8
C5414	Southport	O:II	100	136	9.9	7.0
C5709	Llandough	O:16	100	123	3.8	8.7
C6061	Newcastle upon Tyne	O:16	100	128	10.1	8.5
C6157	Hammersmith	O:16	100	151	10.9	7.7
C6463	Epsom	O:16	100	122	10.5	6.2

NT = Not typable; PA = polyagglutinable (agglutinated with antisera to types O:3, O:9, O:10 and O:13). Pen G = benzylpenicillin; Carb = carbenicillin; Ceph = cephaloridine; Oxa = oxacillin.

TABLE II

*Serology, phage typing and antibiograms of *P. aeruginosa* strains Dalgleish and PU21 and their transconjugants*

Strain	Serotype	Phage susceptibility pattern	MIC (mg/L) of					
			Carbenicillin	Streptomycin	Spectinomycin	Sulphamethoxazole	Kanamycin	Tetracycline
<i>P. aeruginosa</i> Dalgleish	O:16	A	> 2048	512	> 2048	> 1024	32	8
<i>P. aeruginosa</i> Dalgleish (pUZ8)	O:16	A	> 2048	512	> 2048	> 1024	> 256	> 128
<i>P. aeruginosa</i> PU21	O:16	B	32	> 2048	128	128	64	8
<i>P. aeruginosa</i> PU21(pUZ8::PSE-4)	O:16	B	> 2048	> 2048	> 2048	> 1024	> 256	> 128

A = susceptible to phages 21, 68 and 1214; B = susceptible to phages 7, 16, 21, 44, 68, 73, F7, F8, 109, 119X, 1214 and M6.

(pUZ8), *P. aeruginosa* PU21 and *P. aeruginosa* PU21(pUZ8::PSE-4) are given in table II. All these strains were of serotype O:16 but gave weak reactions with antisera to types O:2(a) and O:2(b). *P. aeruginosa* Dalgleish and its (pUZ8)⁺ transconjugant were susceptible to only three phages whereas strains PU21 and PU21(pUZ8::PSE-4) were susceptible to 12. Using strain PU21 (pUZ8::PSE-4) as a donor we transferred the pUZ8::PSE-4 recombinant plasmid into *P. aeruginosa* strains of O serotypes 2, 4, 8, 10 and 15 and in no case was plasmid acquisition associated with changes in serotype or

phage susceptibility. Strain PU21(pUZ8::PSE-4) and other transconjugants with the (pUZ8::PSE-4) plasmid acquired high level resistance to carbenicillin, spectinomycin, sulphonamide, tetracycline and kanamycin (table II). The last two resistances were encoded by plasmid pUZ8, and only the first three were attributable to the PSE-4 coding element. Acquisition of streptomycin resistance could not be monitored in strain PU21 because this strain was intrinsically extremely resistant to this antibiotic. Streptomycin resistance was acquired, however, by recipients when strain PU21(pUZ8::PSE-4) was the donor.

The pUZ8::PSE-4 plasmid was wholly stable in strain PU21 and the O:4, O:8 and O:15 transconjugants during repeated nutrient-broth subculture, but tended to be lost by the O:10 and, surprisingly, the O:2 transconjugants.

DISCUSSION

Sixteen of the 20 PSE-4⁺ strains of *P. aeruginosa* from our survey and from earlier collections of strains reacted with antisera to type O:16 and two with antisera to type O:2(b). These serotypes, together with O:2(a) and O:5, are closely related, sharing a common group antigen (group II), and may be distinguished only by the use of absorbed sera (Pitt, 1981). Moreover, antigenic drift between these types has been observed in isolates from the same source over a prolonged period (Pitt, unpublished observations). The only non-group-II PSE-4⁺ isolates found were a non-typable strain and one which was agglutinated by several unrelated antisera. Because only 9% of *P. aeruginosa* are of serotype O:16 and only 16% have the common group-II antigen (Pitt, 1981) it appears unlikely that this distribution of PSE-4 enzyme was a product of chance ($p < 10^{-7}$). These observations led us to examine further O:16 and group-II *P. aeruginosa* strains for PSE-4 β lactamase and seven out of 30 O:16 strains were found to be enzyme producers. This frequency was significantly higher than that for the whole species in which only 18 PSE-4⁺ organisms were found amongst 1866 isolates (Williams *et al.*, 1984) (χ^2 test: $p < 0.01$). From these observations we conclude that PSE-4 β lactamase is largely, if not wholly, serotype associated in *P. aeruginosa* from British hospitals. This result may not be valid on an international scale because Jouvenot and colleagues (1983) have reported PSE-4 enzyme from *P. aeruginosa* of various serotypes from a single French hospital. However, these authors did not provide precise details of the enzyme's distribution amongst the serotypes.

A likely explanation of our data is that the PSE-4 coding element is restricted in its distribution by its transmissibility. Extrachromosomal DNA was absent from all our PSE-4⁺ isolates and it seems likely that enzyme production was encoded by an element similar or identical to the PSE-4 coding chromosomally-integrating transposon Tn2521 described from Australian isolates of *P. aeruginosa* (Sinclair and Holloway, 1982). All our isolates resembled the Australian organisms in resistance pattern (resistant to carbenicillin, spectinomycin, streptomycin and sulphonamides) and the element transferred from *P. aeruginosa* Dalglish was identical to Tn2521 in having a mol. wt of 4.8×10^6 and in its susceptibility to restriction endonucleases. However, the mechanism of dissemination is not clear and we were unable to obtain conjugative transfers from wild types to strain PU21, which is also serotype O:16. Similar failures to transfer PSE-4 production by conjugation have been reported by other investigators (Sykes and Matthew, 1976; Jacoby and Matthew, 1979; Sinclair and Holloway, 1982;

Jouvenot *et al.*, 1983). Although we transferred PSE-4 enzyme production to strain PU21 by forming a recombinant plasmid between the PSE-4 coding element and the Inc P-1 plasmid pUZ8, we do not consider this finding to be relevant to the present distribution of the enzyme. Such PSE-4 coding Inc P-1 recombinant plasmids are transferable to *E. coli* (Hedges and Matthew, 1979; Jones, unpublished data) whereas PSE-4 enzyme production has never been observed outside *P. aeruginosa*. Recombinant plasmid formation does, however, represent a mechanism by which the PSE-4 coding element may ultimately achieve a wider distribution. It is possible that transfer of the PSE-4 coding element occurs in nature *via* a serotype-specific phage. Transduction of Tn2521 was described by Sinclair and Holloway (1982) and such a mechanism might explain the distribution of the enzyme in nature.

A second explanation of the association between serotype and PSE-4 β lactamase is that enzyme production is essentially non-transmissible and that enzyme producing isolates represent a 'subtype'. This hypothesis predicted greater similarity within PSE-4⁺ *P. aeruginosa* than between group-II PSE-4⁻ strains. However, phage and pyocin typing revealed that group-II strains represented a cluster regardless of β -lactamase production. The only PSE-4 linked characters were resistance to streptomycin, spectinomycin and sulphonamide and these were determined by the PSE-4 coding element itself, at least in the Dalglish strain.

A final possible explanation of the linkage between PSE-4 production and serotype was that the PSE-4 coding element carried serotype determinants. This hypothesis was largely discounted by transfer of the (pUZ8::PSE-4) recombinant plasmid into *P. aeruginosa* strains of various serotypes. None of these organisms exhibited changes in surface characteristics as revealed by sero- and phage-typing. It may still be argued that the PSE-4 coding element only affects serotype once inserted into the chromosome. This possibility was not disproven but seems unlikely since the reported insertion site of Tn2521 (Sinclair and Holloway, 1982) is remote from the genes known to affect serotype (Holloway, Krishnapillai and Morgan, 1979).

In summary, we demonstrated that PSE-4, which is the most common plasmidic β lactamase in *P. aeruginosa*, was largely confined to strains of serotype O:16 and closely related serotypes. This association may reflect limited transmissibility of the PSE-4 coding element, or may indicate that PSE-4⁺ *P. aeruginosa* are a distinct subtype of the species. It is also apparent from our studies that the designation of PSE-4 as a 'plasmid-mediated β lactamase' (Matthew, 1979; Foster, 1983) is misleading. This enzyme has little in common with the other plasmidic β lactamases, which often occur on widely transferable elements. In many ways, the earlier classification of PSE-4 as an unusual chromosomal β lactamase (Sykes and Matthew, 1976) seems more appropriate.

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