Characteristics of *Pseudomonas aeruginosa* in relation to laboratory-induced resistance to gentamicin

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SUMMARY *Pseudomonas aeruginosa* strain C2 was habituated to gentamicin by serial passage in broth containing increasing concentrations of the antibiotic and up to 250 µg/ml. The resistant progenies differed from the parent strain in antibiotic susceptibility to two other aminoglycosides, colonial morphology, lytic phage patterns, phage adsorption, and agglutination with the seven Fisher's antisera. All the progenies failed to grow at 42°C and oxidised glucose in O/F tubes after incubation at 37°C for three days but were catalase- and oxidase-positive. Reversion to the original properties of the parent strain was demonstrated in all cases after 10 serial subcultures in antibiotic-free broth.

During recent years gentamicin has been widely used in the treatment of severe infections caused by Gram-negative bacilli, particularly *Pseudomonas aeruginosa*. Acquired resistance of *Ps. aeruginosa* to gentamicin associated with its clinical use has been reported from various centres (Greene *et al.*, 1973; Eykyn and Phillips, 1975; Seal and Strangeways, 1975).

Many of the clinical isolates of *Ps. aeruginosa* with minimal inhibitory concentration (MIC) values varying from 8 to 100 µg of gentamicin per ml do not contain known forms of gentamicin-modifying enzymes (Bryan and van den Elzen, 1977), while experiments of conjugal mating with gentamicin-resistant strains as donors and a variety of recipients showed no transfer of gentamicin resistance (Holmes *et al.*, 1974).

Enzyme-negative resistant and susceptible strains show differential permeability to gentamicin, and it seems that relatively low gentamicin concentrations may be the selective force for the emergence of permeability-type resistant strains (Bryan and van den Elzen, 1977). This is also supported by the linear relationship between MICs of gentamicin and the amount of surface lipid that has been demonstrated in wild and induced gentamicin-

resistant *pseudomonas* strains (Pechey and James, 1973).

In this paper we describe differences in some characteristics between wild and laboratory-induced gentamicin-resistant strains. These differences interfere with the correct identification of the organism and its epidemiological characteristics and might be encountered also in gentamicin-resistant variants of *Ps. aeruginosa* isolated from patients receiving gentamicin.

Material and methods

BACTERIAL STRAIN

Ps. aeruginosa strain C2 was isolated from the urine samples of a subject with symptoms of bacterial infection and bacterial counts of $> 10^5$ organisms/ml. The MIC of gentamicin was determined by the broth dilution method with doubled dilutions and was found to be 0.4 µg/ml.

Laboratory resistant variants were produced from the sensitive wild strain by repeated passage in broth containing increasing levels of gentamicin and up to 250 µg/ml.

Oxidase test

Colonies grown on yeast extract agar were tested by the Kovacs (1956) method as modified by Rogers (1963).

Oxidation of glucose was performed by the method of Hugh and Leifson (1953).

Production of pigment

Pyocyanin, pyorubin, and fluorescin production was examined on media A and B (King *et al.*, 1954).

Growth at 42°C was examined by inoculating 5 ml of yeast extract broth (Rhodes, 1959). A strain was considered able to grow at 42°C if it could survive three serial transfers (Haynes and Rhodes, 1962).

Disc susceptibility tests were performed according to the procedure of Bauer *et al.* (1966).

Phage typing

The lysis pattern of the wild type strain and its resistant progenies was determined against 21 phages provided by the Public Health Laboratory, Colindale, UK. The routine test dilution of each phage suspension was determined before use. Cultures to be tested were grown in 5 ml Trypticase Soy broth (BBL) overnight at 37°C. Bacterial lawns were prepared by flooding dry Trypticase Soy agar (BBL) plates with the 18-hour trypticase soy broths. After the surface had dried the RTD of each of the 21 phages was placed in a phage applicator and phages were applied to the surface of the inoculated plates. The plates were incubated at 37°C for 18 hours and the lysis pattern was recorded.

Phage adsorption

Phage adsorption to intact cells of wild and laboratory resistant progenies was measured after mixing bacterial cells (approximately 10⁸ viable cells per ml) with phage at a concentration of approximately 10⁶ plaque-forming units per ml at 37°C in a shaker bath. Samples of the adsorption mixture were removed at 10-minute intervals and diluted 10-fold in cold trypticase soy broth. The diluted samples were centrifuged at 6000 *g* for 10 minutes at 4°C to sediment bacteria and adsorbed phage. The supernatant was withdrawn, serial 10-fold dilutions were prepared, and the surviving, infective phage was titred.

Serotyping

Freshly poured Mueller-Hinton agar plates (Difco) were inoculated with the strains to be tested. The plates were incubated for 18 hours at 30°C. The growth was removed with a sterile cotton swab and suspended in 0.15 ml M NaCl. Nine drops of the suspension were placed on a glass slide, and one drop of each of the seven Fisher's antisera (Fisher *et al.*, 1969) was added to one of the seven drops of suspension. The antisera were diluted in 0.15 M NaCl. Physiological saline and normal rabbit serum, which does not contain antibodies to *Ps. aeruginosa*, were added to the two remaining drops as controls. The drops were then mixed with toothpicks, rocked gently for 1 minute, and observed for macroscopic clumping.

Results

Serial transfers of *Ps. aeruginosa* strain C2 in broth containing increasing concentrations of gentamicin resulted in variants growing in the presence of 1 µg, 8 µg, 16 µg, 32 µg, 62 µg, 125 µg, and 250 µg gentamicin per ml.

Growth at 42°C and the biochemical reactions of the parent sensitive strain and its resistant variants are shown in Table 1.

All the variants were not able to grow at 42°C, while glucose oxidation was delayed for three days. The resistant variants did not differ from the parent strain in oxidase and catalase activities.

The parent strain and its resistant variant were tested for their reaction with the seven Fisher's antisera and for their susceptibility to a set of 21 *Ps. aeruginosa* phages (Table 2).

The resistant variants were nonserotypable and were lysed only by phage 188/i, while the parent strain was of immunotype 1 and of phage type 7/68/73/F7/F8/M4/C11/188/i.

In order to establish whether the resistance of the variants to phages was due to loss of phage receptors adsorption experiments were carried out. Phages 7, 68, 73, F7, F8, M4, and C11, which were active against the parent strain, were examined for their adsorption to the viable cells of all the genta-

Table 1 Properties of *Ps. aeruginosa* C2 and its gentamicin-resistant variants

Properties	<i>Ps. aeruginosa</i> C2	Variants grown in different concentrations of gentamicin (µg/ml)						
		1	8	16	32	62	125	250
Growth at 42°C	+	—*	—	—	—	—	—	—
Oxidase	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Glucose oxidation (hours)	+24	+72	+72	+72	+72	+72	+72	+72

*No growth

Table 2 Immunotypes and phage-patterns of *Ps. aeruginosa* C2 and its resistant variants

	<i>Ps. aeruginosa</i> C2	Variants grown in different concentrations of gentamicin ($\mu\text{g/ml}$)						
		1	8	16	32	62	125	250
Immunotype	1	—*	—	—	—	—	—	—
Phage-pattern	7/68/73/F7/F8/M4/C11/188/i	188/i	188/i	188/i	188/i	188/i	188/i	188/i

*No reaction with any of the seven Fisher's antisera.

micin-resistant variants. None of the phages was adsorbed on to cells of any of the variant strains. The Figure shows an adsorption experiment of phage 7 to cells of *Ps. aeruginosa* strain C2 grown in 1 μg gentamicin per ml.

Figure Adsorption of phage 7 to whole cells of *Ps. aeruginosa* propagating strain 7 and *Ps. aeruginosa* C2 grown in 1 μg gentamicin per ml: ●—● phage 7 and *Ps. aeruginosa* 7; ×—× phage 7 and *Ps. aeruginosa* C2 grown in 1 μg gentamicin per ml; ○—○ phage 7 and Trypticase Soy broth.

It is clear that the resistant variant does not adsorb phage 7, while 86% of the plaque-forming units of phage 7 are adsorbed to the cells of the parent strain. Similar results were obtained when the phages were tested with all the other variants.

Pigment production was not affected by the growth of *Ps. aeruginosa* strain C2 in the presence of gentamicin. Subculture of the variants on trypticase

soy agar plates revealed colonies different in morphology from those of the parent strain. The parent colonies were large, wrinkled, and irregular, but the variant colonies were small and smooth with well-defined edges.

The parent strain and the resistant variants were tested for their susceptibility to other antimicrobial agents by the Bauer-Kirby disc method. No differences were observed in the susceptibility pattern of the parent and the variant strains to carbenicillin, polymyxin and colimicin. On the contrary, the gentamicin-resistant variants were also found to be resistant to the two other aminoglycosides tested, kanamycin and streptomycin, while the parent strain was sensitive to both of them.

Ten serial transfers of the variants in gentamicin-free broth resulted in the recovery of strains that were indistinguishable from the parent strain. The revertants exhibited the same colony morphology, phage pattern, and immunotype as the parent strain. They were also able to grow at 42°C, attacked glucose in 24 hours, and were sensitive to gentamicin, kanamycin and streptomycin. The possibility that the variants are not *Ps. aeruginosa* may be ruled out by the fact that they were susceptible to *Ps. aeruginosa* phage 188/i and they were pigment producers.

Discussion

Cross-resistance between gentamicin, kanamycin, and streptomycin has been reported in wild and laboratory derived resistant strains of *Ps. aeruginosa* (Houang and Greenwood, 1977). Comparative tests of clinically sensitive strains and gentamicin-resistant laboratory variants have shown differences in their growth rates and virulence for mice (Weinstein, 1973). The present findings indicate that the laboratory-induced resistance of *Ps. aeruginosa* to gentamicin is accompanied by the loss of ability to grow at 42°C and of the receptor sites for phages. Besides, the resistant variants become nonserotypable by the sera produced against the seven immunologically distinct lipopolysaccharides of the Fisher's scheme. At this point it is interesting to note that clinical isolates of *Ps. aeruginosa* resistant to gentamicin

were nonserotypable, but after repeated subcultures many of them became typable (Greene *et al.*, 1973), and the observation of Seal and Strangeways (1975) that resistant strains of *Ps. aeruginosa* isolated from one patient who was given gentamicin were lysed only by phage 188/i.

Enzyme-negative, gentamicin-resistant isolates of *Ps. aeruginosa* show a reduced permeability to gentamicin (Bryan and van den Elzen, 1977), a view supported by the finding that the amount of surface lipids in laboratory-induced resistant variants is greater than that of the parent sensitive strains. Our results suggest that the differences between sensitive and resistant variants of *Ps. aeruginosa* are not limited to the surface lipids but are more profound and also include changes in other components of the slime layer and/or the lipopolysaccharide.

Purified slime and lipopolysaccharide have been shown to possess receptor-like properties by their inactivation of phages (Bartell *et al.*, 1971; Reese *et al.*, 1974), and the sugar moiety is the receptor site (Lindberg, 1973). The inability of our resistant variants to adsorb the phages may be due to changes of the sugar moiety of the slime and/or the lipopolysaccharide. The nontypability of the resistant variants with Fisher's antisera is another indication that changes may occur in the sugar or protein moieties of the lipopolysaccharide. Because the slime layer is one of the most toxic components of the microorganism, has antiphagocytic activity, and is produced *in vivo* (Dimitracopoulos *et al.*, 1974; Sensakovic and Bartell, 1974), work is now in progress in our laboratory with extracted slime from a laboratory-induced resistant variant in order to find out possible changes in its chemical composition and biological properties.

Li *et al.* (1974) isolated a gentamicin-resistant variant of *Serratia marcescens*, which did not produce DNase and was not susceptible to phages and bacteriocins as was the case with the parent sensitive strain. The emergence of this variant was attributed by the authors to induction by gentamicin.

The resistance of *Ps. aeruginosa* strains isolated *in vivo* is due primarily to R factors (Richmond *et al.*, 1975), though in some cases a mechanism similar to that demonstrated in the laboratory may operate, especially when there is topical or low-dosage use of gentamicin. Our results suggest that the *in-vivo* emergence of such resistant variants may pose problems in the correct identification of the microorganism and its epidemiological fingerprints.

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