Temperature-dependent expression of the chromosomal β -lactamase gene in a strain of *Pseudomonas aeruginosa*

R. G. HEWINSON and W. W. NICHOLS*

Nuffield Department of Pathology and Bacteriology, University of Oxford and *Regional Public Health Laboratory, Level 6/7, John Radcliffe Hospital, Oxford, OX3 9DU

Summary. A strain of *Pseudomonas aeruginosa* (3-Post) was resistant to cefsulodin and ceftazidime at 37°C but sensitive at 20°C. Resistance was mediated by chromosomally-encoded β lactamase which was synthesised at a high level during growth above 30°C but at a low, inducible level during growth below 27°C.

Introduction

The regulation of chromosomally-encoded β -lactamase synthesis in *Pseudomonas aeruginosa* is an important area of study because resistance to “anti-pseudomonal” β -lactam antibiotics (Slack, 1981) can arise by mutation to constitutive synthesis (King *et al.*, 1983; Williams *et al.*, 1984). This class C β lactamase (Ambler, 1980; Jaurin and Grundstrom, 1981; Knott-Hunziker *et al.*, 1982) is normally inducible by β -lactam compounds (Nordström and Sykes, 1974). Induction of β -lactamase synthesis in *Citrobacter freundii*, apparently involves a positive control mechanism requiring two protein-encoding regulatory genes, termed *ampR* and *ampD* (Lindberg *et al.*, 1985; Lindberg and Normark, 1986). Here we describe a mutant of *P. aeruginosa* that displays properties consistent with the operation of a similar control system.

Materials and methods

Bacteria and sensitivity testing

Organisms used were *P. aeruginosa* strain 3-Pre, and a cefsulodin-resistant mutant derived from it *in vitro*, strain 3-Post (Slack and Pitt, 1982; Slack *et al.*, 1983). MIC values were determined on Isosensitest Agar (Oxoid) by the plate-incorporation method, with an inoculum of $c. 10^4$ cfu/spot. Disk tests were performed by Stokes's method on Isosensitest Agar.

Growth and harvesting of bacteria

Cells were grown in 80-ml volumes of the chemically-defined medium (pH 7.8) of Anwar *et al.* (1983) in 250-

ml conical flasks with baffles, orbitally shaken at 170 rpm (32-mm diameter of orbit). Cells were harvested at $E_{650} = 0.2$ (0.098 mg dry wt cells/ml) which represented the early exponential phase of growth. Induction of β lactamase was achieved by adding benzyl penicillin (200 mg/L final concentration) to the culture 2 h before harvesting the cells by centrifugation at 9400 g (r_{av} 7.0 cm) for 10 min at 4°C. The supernate was discarded. For suspensions to which penicillin had not been added, the pellet was used as the source of β lactamase. For induced cells, the first pellet was washed by resuspension in 35 ml of ice-cold 5 mM $MgCl_2$ –25 mM 3-(N-morpholino)-propanesulphonic acid (MOPS) (pH 7.4) and re-centrifugation as above. The pellet of washed bacteria was used as the source of β lactamase from induced cells.

Liberation of β lactamase

Pellets were subjected to three cycles of freezing and thawing, resuspended in ice-cold 5 mM $MgCl_2$ –25 mM MOPS (pH 7.4) and subjected to a further freeze-thaw cycle. This treatment should release more than 90% of the β lactamase of *P. aeruginosa* (Berks, 1977). A sample of the final freeze-thawed preparation of cells was centrifuged at 11000 g (r_{av} 9 cm) for 34 min at 4°C. β -Lactamase activity was assayed in the supernate.

Assays of β -lactamase activity

The hydrolysis of cephalosporin C was monitored spectrophotometrically at 260 nm at 37°C in 5 mM $MgCl_2$ –25 mM MOPS (pH 7.4). The kinetic parameters, K_m and specific V_{max} , were determined by automated (Nichols and Hewinson, 1987) half-time analysis of reaction progress curves (Wharton and Szawelski, 1982).

Results

As defined by disk-diffusion tests, *P. aeruginosa* 3-Post was sensitive to cefsulodin and ceftazidime

at 20°C but was resistant at 37°C. *P. aeruginosa* 3-Pre, from which strain 3-Post was derived (Slack and Pitt, 1982), was sensitive to both antibiotics at both temperatures. The MIC of cefsulodin at 37°C for strain 3-Pre was 1.70–1.75 mg/L, and for strain 3-Post was 17–18 mg/L. However, at 17°C the MIC of cefsulodin for both strains was 0.25–0.5 mg/L.

The table shows the effects of growth temperature on β -lactamase synthesis in the two strains. The β lactamase of strain 3-Post was synthesised at an elevated level at 37°C but at a 14-fold lower level at 21°C. The lower basal level (480 nmol/min/mg dry wt) was higher than that of the parent strain, 3-Pre, at either temperature (24 and 72 nmol/min/mg dry wt at 22°C and 37°C respectively). When strain 3-Post was grown at the lower temperature, β -lactamase synthesis was inducible.

The change from low-level to high-level β -lactamase synthesis in strain 3-Post occurred between the growth temperatures 27°C and 29.5°C (figure). The amount of β lactamase synthesised in strain 3-Post decreased with rising temperature above 30°C.

Discussion

The different cefsulodin and ceftazidime sensitivities of *P. aeruginosa* 3-Post at room temperature and at 37°C were, at least partly, determined by the levels of β -lactamase synthesised at these two temperatures. We suggest that a protein involved in regulating β -lactamase synthesis is thermolabile in strain 3-Post, and that this explains the sharpness of the temperature dependence of the cell complement of β -lactamase (figure).

Figure. β -Lactamase activities of extracts of *P. aeruginosa* 3-Post grown at different temperatures. Assays of cephalosporin C hydrolysis were all made at 37°C. The enzyme kinetic parameter, specific $V_{\max}$, was determined for each point by half-time analysis of reaction progress curves. Error bars show ± 2 SEM determined from four to six replicate measurements. At 27°C and below, error bars were too small to draw.

In *C. freundii* there is a regulatory protein (the *ampR* gene product) that represses synthesis of the β lactamase about 2.5-fold but that apparently acts as an activator of transcription of the β -lactamase

Table. β -Lactamase activities (substrate cephalosporin C) assayed at 37°C

Strain	Mean growth temperature (°C)	Induced*/uninduced	K_m (μM)		Specific $V_{\max}$ (nmol/min/mg dry-wt)		n
			mean	SEM	mean	SEM	
3-Pre	21.9	uninduced	18.2	1.8	24	2	3
		induced	25.5	2.6	1260	200	6
3-Pre	37.0	uninduced	20.0	1.1	72	3	4
		induced	18.9	1.3	1240	40	4
3-Post	21.3	uninduced†	19.4	1.0	480	20	5
		induced	17.2	0.7	1490	40	5
3-Post	37.0	uninduced†	21.3	1.2	6560	240	4

* β Lactamase was induced by the addition of benzyl penicillin (200 mg/L) to the culture 2 h before harvesting the cells.

† The data for uninduced cells of 3-Post are included in the figure.

(*ampC*) gene on induction (Lindberg *et al.*, 1985; Lindberg and Normark, 1986). Mutations to constitutive synthesis map outside the *ampR-ampC* region but the constitutive phenotype is only observed in the presence of a functional *ampR* gene product (Lindberg *et al.*, 1985). The results of the experiments reported in the present communication are consistent with this system also operating in *P. aeruginosa* because a mutant of *C. freundii* has been isolated which demonstrates temperature-sensitive expression of β lactamase with a phenotype that is very similar to that of strain 3-Post (Sawai *et al.*, 1977).

Constitutive synthesis of the class C β lactamase

in *P. aeruginosa* confers resistance to cefsulodin and ceftazidime whereas basal, but inducible, synthesis does not (Livermore, 1986). The mechanism of this type of resistance has been the subject of debate as to whether hydrolysis (Livermore, 1985) or non-hydrolytic "trapping" (Sanders, 1984) by periplasmic β lactamase is responsible. The mechanism of resistance of strain 3-Post to cefsulodin at 37°C could be best explained by β -lactamase-catalysed hydrolysis of the antibiotic within the periplasmic space (Hewinson *et al.*, 1985).

We thank the EPA Research Trust for a scholarship for training in research methods for R.G.H.

REFERENCES

- Ambler R P 1980 The structure of β -lactamases. *Philosophical Transactions of the Royal Society of London Series B* **289**: 321-331.
- Anwar H, Lambert P A, Brown M R W 1983 Effect of divalent metal ions on the production of outer membrane protein H₂ in mucoid and non-mucoid strains of *Pseudomonas aeruginosa*. *FEMS Microbiology Letters* **16**: 247-250.
- Berks M 1977 Studies on β -lactamases from *Pseudomonas aeruginosa*. DPhil Thesis, University of Oxford.
- Hewinson R G, Slack M P E, Nichols W W 1985 A quantitative study of beta-lactamase-mediated trapping *vs* hydrolysis in cefsulodin-resistant *Pseudomonas aeruginosa*. In: Ishigami J (ed) Recent advances in chemotherapy, antimicrobial section. University of Tokyo Press, Tokyo, pp 365-366.
- Jaurin B, Grundström T 1981 *ampC* cephalosporinase of *Escherichia coli* K-12 has a different evolutionary origin from that of β -lactamases of the penicillinase type. *Proceedings of the National Academy of Sciences of the USA* **78**: 4897-4901.
- King A, Shannon K, Eykyn S, Phillips I 1983 Reduced sensitivity to β -lactam antibiotics arising during ceftazidime treatment of *Pseudomonas aeruginosa* infections. *Journal of Antimicrobial Chemotherapy* **12**: 363-370.
- Knott-Hunziker V, Petursson S, Jayatilake G S, Waley S G, Jaurin B, Grundström T 1982. Active sites of β -lactamases. The chromosomal β -lactamases of *Pseudomonas aeruginosa* and *Escherichia coli*. *Biochemical Journal* **201**: 621-627.
- Lindberg F, Westman L, Normark S 1985 Regulatory components in *Citrobacter freundii ampC* β -lactamase induction. *Proceedings of the National Academy of Sciences of the USA* **82**: 4620-4624.
- Lindberg F, Normark S 1986 Contribution of chromosomal β -lactamases to β -lactam resistance in enterobacteria. *Reviews of Infectious Diseases* **8** Suppl 3: S292-S304.
- Livermore D M 1985 Do β -lactamases 'trap' cephalosporins? *Journal of Antimicrobial Chemotherapy* **15**: 511-514.
- Livermore D M 1986 Class I β -lactamase expression in *Pseudomonas aeruginosa* and cephalosporin resistance. *Lancet* **i**: 450.
- Nichols W W, Hewinson R G 1987 Rapid and automated measurement of K_m and specific V_{max} values of β -lactamases in bacterial extracts. *Journal of Antimicrobial Chemotherapy* **19**: 285-295.
- Nordström K, Sykes R B 1974 Induction kinetics of β -lactamase biosynthesis in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **6**: 734-740.
- Sanders C C 1984 Inducible β -lactamases and non-hydrolytic resistance mechanisms. *Journal of Antimicrobial Chemotherapy* **13**: 1-3.
- Sawai T, Nakajima S, Morohoshi T, Yamagishi S 1977 Thermolabile repression of cephalosporinase synthesis in *Citrobacter freundii*. *Microbiology and Immunology* **21**: 631-638.
- Slack M P E 1981 Antipseudomonal β -lactams. *Journal of Antimicrobial Chemotherapy* **8**: 165-170.
- Slack M P E, Pitt T L 1982 Characterization of cefsulodin-resistant variants of *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy* **9**: 111-117.
- Slack M P E, Nichols W W, West C 1983 Studies on the mechanisms of cefsulodin resistance in *Pseudomonas aeruginosa*. In: Spitzky K H, Karrer K (eds) Proceedings of the thirteenth international congress of chemotherapy, Part 87: Antibiotic resistance in vitro. Verlag H. Egermann, Vienna, pp 40-43.
- Wharton C W, Szawelski R J 1982 Half-time analysis of the integrated Michaelis equation. Simulation and use of the half-time plot and its direct linear variant in the analysis of some α -chymotrypsin-, papain- and fumarase-catalysed reactions. *Biochemical Journal* **203**: 351-360.
- Williams R J, Livermore D M, Lindridge M A, Said A A, Williams J D 1984 Mechanisms of beta-lactam resistance in British isolates of *Pseudomonas aeruginosa*. *Journal of Medical Microbiology* **17**: 283-293.