Production of a variant of β -lactamase II with selectively decreased cephalosporinase activity by a mutant of *Bacillus cereus* 569/H/9

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1. Mutants of *Bacillus cereus* 569/H/9 have been screened in a search for strains that synthesize variants of β -lactamase II. 2. One of these mutants (strain 569/H/9/1) produces a β -lactamase II-like enzyme that shows a selective decrease in cephalosporinase activity. 3. β -Lactamase II from strain 569/H/9/1 has been purified to apparent homogeneity and its kinetic properties have been examined. This enzyme resembles the parent β -lactamase II in its relative activity with benzylpenicillin as substrate when Zn(II) is replaced by other metal ions, but differs detectably from the parent enzyme in its isoelectric point.

β -Lactamase II, one of the two extracellular β -lactamases (EC 3.5.2.6) produced by *Bacillus cereus* 569/H/9, hydrolyses the β -lactam ring of penicillins and many cephalosporins at similar rates. Since the initial recognition of this enzyme (Newton & Abraham, 1956), and its subsequent purification to apparent homogeneity (Davies *et al.*, 1974), interest has centred on its requirement, unique amongst the known β -lactamases, for a metal ion for activity (Sabath & Abraham, 1966). Its activity, which is highest with the zinc(II) enzyme, parallels the binding of the first of two metal ions to the enzyme (Davies & Abraham, 1974). This binding site has been shown by u.v.-absorption spectroscopy (Davies & Abraham, 1974) and n.m.r. spectroscopy (Baldwin *et al.*, 1978, 1979) to contain one cysteine and three histidine residues.

In contrast with this information about the nature of the metal-binding site, almost nothing is known about the identity of the amino-acid residues involved in catalysis. To throw light on this question it was decided to attempt to isolate mutants of *B. cereus* 569/H/9 that produced variants of β -lactamase II with altered catalytic properties. Characteristics of the β -lactamase II from one such mutant (strain 569/H/9/1) are described in the present paper. The mutation differed in kind from one that occurred spontaneously in *B. cereus* 5/B, since the latter resulted in a selective and total loss of ability to produce β -lactamase I (Davies *et al.*, 1975).

Materials and methods

Organism

B. cereus 569/H/9 is a constitutive β -lactamase-producing strain kindly provided by Professor M. R. Pollock. Its pedigree is described by Davies *et al.* (1974).

Reagents

Nutrient agar, nutrient broth no. 2, and Lab-lemco were all from Oxoid, Ware Rd., Basingstoke, Hants., U.K. Bacteriological peptone was supplied by Evans Medical, Speke, Liverpool, U.K. Ethylmethanesulphonate was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Acid fuchsin (special grade for Andrade's indicator) was from Hopkin and Williams, Chadwell Heath, Essex, U.K.

Iodo[2-¹⁴C]acetic acid (sp. radioactivity 57 Ci/mol) was from The Radiochemical Centre, Amersham, Bucks., U.K., and was dissolved in 250 μ l of 200 mM-iodoacetic acid (re-crystallized from light petroleum, b.p. 60–80°C) to give a solution of specific radioactivity 0.98 Ci/mol. Guanidinium chloride (AristaR grade) was from BDH Chemicals, Poole, Dorset, U.K., cycloserine was from Sigma Chemical Co., Poole, Dorset, U.K., and benzylpenicillin and cephalosporin C were from Glaxo Research Laboratories, Greenford, Middx., U.K. All other chemicals were of AnalaR grade.

Media

Nutrient agar and nutrient broth were prepared by dissolving 28 g and 25 g respectively in 1 litre of water. S-broth (Pollock, 1953) contained 10 g of Evans peptone, 3 g of Lab-lemco and 2 g of NaCl in 1 litre of water, and its pH was adjusted to 7.0 with 10 M-NaOH before autoclaving. S-broth/citrate was as for S-broth with the addition of 7 g of trisodium citrate dihydrate per litre; S-broth/citrate/zinc was the same as S-broth/citrate except that it contained 1 mM-ZnSO₄; S-agar/zinc was made by addition of agar (15 g/litre) to S-broth/citrate/zinc before autoclaving, and S-agar/zinc/Andrade's indicator was prepared as for S-agar/zinc, except that 4% (v/v) of indicator solution (prepared by dissolving 0.5 g of acid fuchsin in 100 ml of water and adding 17 ml of 1 M-NaOH before autoclaving) was added to the molten agar immediately before the plates were poured.

Assay of β -lactamase in solution

Unless otherwise stated assays for β -lactamase I and II activity were as described by Davies *et al.* (1974), except that those for β -lactamase II were carried out at pH 6.0.

Preparation of spore suspensions

A single colony on an agar plate was used to inoculate a nutrient agar slope that, after 24 h at 30°C, provided inoculum for 100 ml of S-broth in a shake-flask. After incubation at 30°C for 48 h with shaking (180 rev./min) the spores were isolated by centrifugation, washed twice with water and then resuspended in 20 ml of water. The resuspension was heated at 60°C for 60 min, to kill any vegetative cells, and stored at 4°C.

Mutagenesis

A suspension of spores of *B. cereus* 569/H/9, in 0.1 M-sodium phosphate buffer, pH 7.0 (10 ml; 10⁸ spores/ml), was divided in two and each half was shaken at 27°C for 30 min in a conical flask on a rotary shaker (220 rev./min). Ethylmethanesulphonate was added to one suspension to give a final concentration of 0.2 M and shaking was continued for 30 min or 20 h. The spores from both suspensions were then washed twice by centrifugation and re-suspension in the phosphate buffer, and finally re-suspended in 5 ml of water. Appropriate dilutions from both suspensions were plated on S-agar/zinc to assess viability, which for the treated suspension after 20 h was typically 8% of the control. A sample of each suspension (0.25 ml) was added to 10 ml of nutrient broth and incubated for 16 h at 30°C to permit expression of any mutations carried. The undiluted culture was plated on S-agar/zinc containing 150 µg of cycloserine/ml to assess mutagenesis, and 10⁵-fold dilutions of the

culture were plated on S-agar/zinc/Andrade's indicator to examine β -lactamase production by colonies. After 20 h treatment with the mutagen the frequency of cycloserine-resistant cells had increased 100-fold from a spontaneous level of 10⁻⁶, whereas the frequency of β -lactamase-negative cells was less than 10⁻².

Selection of β -lactamase-negative mutants

The procedure used for detection of β -lactamase-negative colonies was based on the method of Manson *et al.* (1954). About 200 viable cells were plated on S-agar/zinc/Andrade's indicator and incubated at 30°C for 12 h. The plates were then sprayed evenly with just enough of one of the following solutions to visibly moisten the surface of the agar and colonies. To detect β -lactamase I activity the solution contained 1 g of benzylpenicillin K salt in 5 ml of 10 mM-EDTA, pH 7.0; to detect β -lactamase II activity 1.2 g of cephalosporin C was dissolved in 5 ml of 100 mM-ZnSO₄, pH unadjusted. Colonies producing β -lactamase became the centre of a bright red area in about 5 min, and for up to 15 min could be distinguished clearly from colourless non-producing colonies. Colonies that were clearly less coloured than the majority after treatment with one or other of the two sprays were picked off into nutrient broth and the broth was incubated at 30°C for 16 h. Platings were then made on to three S-agar/zinc/Andrade's indicator plates, on one of which colonies were tested for β -lactamase I activity, and on one for β -lactamase II activity. If the apparent difference in the two activities was markedly greater than that observed with the parent strain, a colony was taken from the third plate into nutrient broth. The process was repeated until the isolate had shown a consistent phenotype and lack of contamination over three cycles. To assess β -lactamase production by an isolate, S-broth/citrate/zinc (100 ml) in a 500 ml conical flask was inoculated with 1 ml of an overnight nutrient broth culture of the strain and shaken (170 rev./min) at 30°C for 12 h. The culture filtrate was then assayed for β -lactamase I and β -lactamase II.

Two further screening procedures were used to eliminate β -lactamase-negative mutants in which loss of a particular activity was caused by failure to produce the corresponding protein. In the first method the exocellular proteins produced by mutant strains of *B. cereus* were examined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969). A shake-flask culture grown as described above was made 10 mM in EDTA (to inactivate metal ion-dependent proteinases) and centrifuged at 4°C to remove the cells. The supernatant was dialysed against 1 mM-EDTA at 4°C and freeze-dried. A

sample (2–3 mg) of this material was subjected to gel electrophoresis.

In the second method, based on that of Pollock (1964), antiserum prepared in rabbits against samples of pure β -lactamase I and β -lactamase II were used. There was no cross reaction between β -lactamase I and the antiserum to β -lactamase II or vice versa.

Large-scale growth of B. cereus 569/H/9 and mutant 569/H/9/1

Three 500 ml conical flasks, each containing 100 ml of S-broth/citrate, were each inoculated with 0.1 ml of a spore suspension of the parent or mutant strain (10^8 spores/ml), prepared as described by Kuwabara (1970), and incubated at 30°C for 6 h with shaking (140 rev./min). These cultures were used to inoculate 15 litres of S-broth/citrate/zinc in a 20-litre fermenter (L. H. Engineering, Stoke Poges, Bucks., U.K.). Cells were grown at 30°C with stirring (800 rev./min) and continuous aeration (15 litres/min). Foaming was minimized by automatic addition of poly(propylene glycol) 2025. Hourly measurements of β -lactamase I and II activity, and of absorption, were made (Davies *et al.*, 1974), until β -lactamase II activity no longer increased (usually after approx. 8 h). The culture was then harvested by centrifugation and β -lactamases I and II were isolated from the supernatant as described by Davies *et al.* (1974), except that the β -lactamase II pool from the Sephadex CM-50 column was concentrated by stepwise elution from a small column of Sephadex CM-50 as follows. The pool, containing up to 150 mg of β -lactamase II, was dialysed against 4 $\times$ 8 litres of 1 mM-ZnSO₄, mixed with 0.1 vol. of 1 M-Tris/HCl, 1 mM-ZnSO₄, pH 7.0, and passed through 2 ml of Sephadex CM-50, previously equilibrated with 100 mM-Tris/HCl, 1 mM-ZnSO₄, pH 7.0, in a Pasteur-pipette column. The enzyme was eluted with the latter buffer containing 1 M-NaCl, and appeared in the first 10 ml of the eluate.

The amount of β -lactamase II produced in the S-broth/citrate/zinc medium was about twice that obtained with the modified CH/C medium of Kuwabara (1970), but the production of β -lactamase I was similar in both media.

Isoelectric focusing

Samples (20 μ g) of β -lactamases I and II were subjected to isoelectric focusing in polyacrylamide gels (LKB Ampholine Wide-range PAG plates, 1804-101, pH 3.5–9.5; LKB Instruments, South Croydon, Surrey, U.K.).

Results and discussion

Characteristics of β -lactamase-negative mutants

Since the aim of the present work was to isolate and characterize mutant strains of *B. cereus* 569/

H/9 that would carry single-point substitution mutations in the genes coding for β -lactamase I or β -lactamase II, ethylmethanesulphonate, an alkylating agent that generally produces non-clustered G-C $\rightarrow$ A-T transitions (Bridges, 1976), was chosen. After ethylmethanesulphonate-induced mutagenesis, two strains with β -lactamase I activity decreased or absent, but with normal β -lactamase II activity, and five strains with β -lactamase II activity decreased or absent, but with normal β -lactamase I activity, were isolated from a total of 58 000 colonies screened on plates containing Andrade's indicator as described in the Materials and methods section. Of these seven strains, six were shown to lack the corresponding β -lactamase protein by gel electrophoresis in sodium dodecyl sulphate and by failure to react to antisera raised against the 569/H/9-type enzyme. The remaining strain, isolated after treatment for 20 h with ethylmethanesulphonate and designated 569/H/9/1, produced normal activities of β -lactamase I and of both β -lactamase proteins, but too little β -lactamase II activity for this to be observed by the colorimetric test used for detecting β -lactamase production by isolated colonies.

Isolation of β -lactamases I and II from mutant B. cereus strain 569/H/9/1

Attempts to determine the frequency of reversion during the growth of the mutant strain 569/H/9/1 were unsuccessful, because it was able to grow in concentrations of cephalosporin C as high as 3 mg/ml. β -Lactamase I and β -lactamase II from strain 569/H/9/1 were isolated from the culture supernatant by adsorption on Celite followed by chromatography on Sephadex CM-50 (Davies *et al.*, 1974). The final β -lactamase II 569/H/9/1 pool was heated at 60°C for 60 min (91% recovery) to remove any residual β -lactamase I, and concentrated on a small column of Sephadex CM-50. The final yields of β -lactamase I and β -lactamase II from strain 569/H/9/1 were 125 mg and 97 mg respectively. Both β -lactamases resembled the corresponding enzymes from the parent strain in their behaviour during purification, but the activity of β -lactamase II from strain 569/H/9/1 with cephalosporin C as substrate was only about 2.5% of that of β -lactamase II from strain 569/H/9.

Physical and chemical properties of β -lactamases I and II from strain 569/H/9/1

The purified samples of β -lactamases I and II from strain 569/H/9/1 were examined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969). Both preparations appeared to be homogeneous and their mobilities appeared to be identical with those of the corresponding two enzymes from the parent strain.

The patterns obtained when purified samples of β -lactamases I and II from strain 569/H/9/1 were examined by isoelectric focusing in polyacrylamide gels are shown in Fig. 1. Whereas the samples of β -lactamase I had the same isoelectric point as the major component of β -lactamase I isolated from the parent strain, the sample of β -lactamase II separated into two components, of which the major one had a detectably higher isoelectric point than any of the multiple components of β -lactamase II isolated from the parent strain.

β -Lactamase II from strain 569/H/9/1 still binds at least one atom of zinc(II). After dialysis against 5 mM-NH₄HCO₃, pH 7.0, containing 41.2 μ M-ZnSO₄, estimations by atomic-absorption spectroscopy gave concentrations of Zn(II) of 88.6 μ M in the solution of protein whose concentration was 47.4 μ M (by weight). In addition, the mutant enzyme appears to retain similar thermostability to the enzyme from the parent strain (Davies *et al.*, 1974).

The mutant β -lactamase II also cross-reacts with rabbit antisera raised against pure β -lactamase II from the parent strain. In double-diffusion immunoprecipitation experiments a reaction of identity was observed between the two enzymes.

The amino-acid composition of the β -lactamase II from strain 569/H/9/1 (Table 1) is very similar to those reported by Davies *et al.* (1974) and Thatcher (1975) for β -lactamase II from the parent strain 569/H/9.

When β -lactamase II from the mutant was allowed to react with sodium iodo[2-¹⁴C]acetate in guanidinium chloride (Davies *et al.*, 1974; Baldwin *et al.*, 1979) the incorporations of 0.69 and 0.70 mol of ¹⁴C/mol of enzyme was observed on two separate occasions, in good agreement with the values obtained with the parent strain (0.7 mol of ¹⁴C/mol). Thus it appears that the mutant β -lactamase II, like that from strain 569/H/9, contains a single thiol group.

Kinetic properties of β -lactamases I and II from strain H/569/H/9/1

The specific activity of the purified β -lactamase I from the mutant 569/H/9/1 did not differ significantly from that of the β -lactamase I from the parent strain.

Direct comparison of the relative activities of the preparations of β -lactamase II from the two strains showed that the quotient [rate of hydrolysis of cephalosporin C (6 mM)]/[rate of hydrolysis of benzylpenicillin (2 mM)] was 1.1 for the enzyme from the parent and 0.05 for the enzyme from the mutant. A more detailed comparison of the substrate profiles for β -lactamase II from the mutant strain 569/H/9/1 and the parent strain is given in Table 2. It is apparent that the values for k_{cat} , obtained for the

Fig. 1. Isoelectric focusing of β -lactamases I and II from the parent strain 569/H/9 and mutant 569/H/9/1. Isoelectric focusing was in polyacrylamide gel with 20 μ g samples of enzyme. The major component is in each case indicated by the solid line. Samples (1) and (2) are β -lactamase II from the mutant and parent strains respectively, and samples (3) and (4) are β -lactamase I from the mutant and parent strains respectively. The bands are proteins detected by staining with Coomassie Blue.

Table 1. The amino-acid compositions of β -lactamase II from mutant 569/H/9/1 and the parent strain 569/H/9. Amino-acid compositions were determined as described by Baldwin *et al.* (1979). Cysteine was estimated as described in the text.

	Composition of β -lactamase II (nmol/nmol) from:		
	Mutant 569/H/9/1	Parent	
		*	†
Asx	25.4	25	24
Thr	13.8	12	15
Ser	11.0	10	10
Glx	17.8	18	18
Pro	5.2	7	5
Gly	16.2	18	19
Ala	10.9	10	11
Val	17.4	22	20
Met	1.9	2	2
Ile	10.1	11	11
Leu	22.8	23	23
Tyr	4.6	4	5
Phe	3.6	3	4
His	6.6	5	6
Lys	18.8	19	19
Arg	5.7	4	5

* Davies *et al.* (1974).
† Thatcher (1975).

Table 2. Kinetic constants for β -lactamase II from the mutant strain 569/H/9/1 and the parent strain 569/H/9. Assays were carried out as described in the Materials and methods section, in 1M-NaCl/0.3mM-ZnSO₄ at 30°C and pH 6.0.

Substrate	K_m (mM)		$10^{-3} \times k_{cat}$ (min ⁻¹)		k_{cat}/K_m for parent/mutant	$k_{cat}^\dagger$ for parent/mutant
	Mutant	Parent	Mutant	Parent		
Cephalosporin C	0.14	0.4*	1.0	40*	13	95
Cephaloridine	2.68	3.9*	4.4	47*	7.3	27
Cephalothin	0.15	0.2*	2.2	56*	19	63
Benzylpenicillin	9.57	1.45	173	69	2.6	(1)
Methicillin	2.20	1.80	105	134	1.6	3.3

* M. Brightwell, personal communication.

† Relative to quotient for benzylpenicillin.

Table 3. The effects of metal ion replacement on activity for β -lactamase II from the mutant strain 569/H/9/1 and the parent strain 569/H/9

Assays were carried out with 10mM-benzylpenicillin as substrate and 1mM metal ion in 1M-NaCl. Apoenzyme was prepared by treatment of the zinc-enzyme with 10mM-EDTA for 16h at 4°C, pH 7.0. N.D. signifies that no activity was detected.

Metal ion	Activity (relative) of enzyme from:	
	Mutant 569/H/9/1	Parent 569/H/9
Zn(II)	100	100
Co(II)	10.1	12.6
Mn(II)	4.4	6.7
Cd(II)	1.3	1.0
Hg(II)	N.D.	0.7

mutants of *Bacillus licheniformis* (Pollock, 1968); similarly, an increase was accomplished with mutants of *Escherichia coli* RTEM β -lactamase under selection pressure (Hall & Knowles, 1976). The changes in the structures of the proteins are not known in these instances. The enzyme from mutants of *Staphylococcus aureus*, on the other hand, have been more fully characterized structurally than kinetically (Novick, 1963; Richmond, 1963; Ambler, 1980).

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enzyme from the mutant with several cephalosporins as substrates are less than 10% of the values for the enzyme from the parent strain, whereas with benzylpenicillin and methicillin as substrates they are similar to the values for the parent.

The β -lactamase II from the mutant strain resembles that from the parent in that maximum activity with either benzylpenicillin or cephalosporin C as substrate is dependent on the presence of zinc(II). The activity in the presence of 10mM-EDTA is less than 0.1% of that in the presence of Zn(II) with benzylpenicillin as substrate. The effects of the replacement of Zn(II) by other metal ions on the rate of hydrolysis of benzylpenicillin by β -lactamase II from strain 569/H/9/1 and the parent strain respectively are given in Table 3. Similar changes were observed with each enzyme.

Comparisons with other β -lactamases

The selective decrease in activity towards certain cephalosporins described in the present paper may be contrasted with the relative increase in this activity that was observed for the β -lactamase from

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