The Purification and Properties of the β -Lactamase Specified by the Resistance Factor R-1818 in *Escherichia coli* and *Proteus mirabilis*

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1. The β -lactamase specified by the R-1818 resistance factor in *Escherichia coli* was purified 300-fold; the resulting preparation gave a single peak on Sephadex G-100 and a single band on polyacrylamide-gel electrophoresis. 2. The β -lactamase specified by the same R-factor in *Proteus mirabilis* was purified over 2000-fold, but was still far from pure. The specific activity of this preparation was one-fifth that of the purified enzyme from *E. coli*. 3. The two enzymes were shown to be identical as regards substrate specificity, pH optimum, K_m values and molecular weight. 4. It is suggested that the low β -lactamase activity of extracts of *P. mirabilis* (R-1818), about 5% of that from *E. coli* (R-1818) in crude extracts, could be due to inefficient transcription of the R-factor DNA by *Proteus* RNA polymerase.

It has been shown for several R-factors that the activity of β -lactamase [penicillinase, penicillin (cephalosporin) β -lactam amidohydrolase, EC 3.5.2.6] specified by a particular R-factor is similar for a number of host species: *Escherichia coli*, *Salmonella typhi*, *Klebsiella aerogenes*, *Serratia marcescens*, and *Alkalescens* (Datta & Kontomichalou, 1965; Kontomichalou, 1967; Smith, 1969). Smith (1969) showed that when R-1818 was introduced into two strains of *Proteus mirabilis* the activity of β -lactamase was only 6% of that from *E. coli* (R-1818). Similar results were obtained with the R-factor R-7268 (otherwise known as R-1) (Smith, 1969); this is in direct conflict with the results reported by Falkow, Haapala & Silver (1969) who claimed to have shown that the activities of β -lactamase and chloramphenicol acetylase (acetyl-CoA-chloramphenicol acetyltransferase) in *P. mirabilis* (R-7268) were 7-10 times those found in *E. coli* (R-7268). The reason for this discrepancy is not yet known.

The results obtained by Smith (1969) can be accounted for in two ways: either *P. mirabilis* (R-1818) produces a lower amount of an enzyme which is identical with that produced by the other strains, or the R-factor β -lactamase is modified in some way so as to possess much lower specific activity. (A third possibility is that some combination of both effects is involved.) As part of an attempt to elucidate the reason for the lowered activity in *P. mirabilis*, it was necessary to purify and investigate the properties of the β -lactamase

specified by the R-1818 R-factor when harboured by *E. coli* and *P. mirabilis*.

MATERIALS

Bacterial strains. *E. coli* laboratory number 210 was *E. coli* J6-2 (Smith, 1969) containing a high-activity β -lactamase-producing mutant of R-1818. *P. mirabilis* laboratory number 161 was *P. mirabilis* F67 (Smith, 1969) into which this mutant R-factor has been transferred.

Drugs and chemicals. Sodium ampicillin, sodium benzylpenicillin, disodium carbenicillin, sodium methicillin and cephaloridine were obtained commercially. Propicillin, penicillin V, phenethicillin (all as potassium salts), sodium cloxacillin, 6-aminopenicillanic acid, cephalothin (sodium salt) and cephalosporin C (potassium salt) were all donated by Beecham Research Laboratories Ltd., Brockham Park, Betchworth, Surrey, U.K. Quinacillin (disodium salt) was a gift from Boots Pure Drug Co. Ltd., Nottingham, Notts., U.K., and sodium oxacillin was generously donated by Bristol Laboratories, Syracuse, N.Y., U.S.A.

Sephadex and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; bovine serum albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.; ovalbumin, cytochrome c and protamine sulphate (ex herring) were purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and chymotrypsinogen, hydrolysed starch for electrophoresis, ammonium sulphate (enzyme grade) and DEAE-cellulose (DE22, Whatman) from British Drug Houses, Poole, Dorset, U.K. Guanidine hydrochloride ('ultra pure') was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Other reagents were analytical grade from BDH Chemicals Ltd. or Koch-Light.

Deionized distilled water was used throughout. Unless otherwise indicated in the text, all buffers were prepared as described by Dawson & Elliott (1959) or Long (1961). Buffer A is 0.025M-sodium phosphate, pH 7.4.

METHODS

β -Lactamase assays. (a) Hydroxylamine. β -Lactamase was normally assayed by the method of Boxer & Everett (1949) as modified by J. T. Smith (unpublished work). The stock solutions used are: 5M-hydroxylamine (hydroxylammonium chloride); 'alkaline buffer', 0.25M-sodium acetate containing 173g of NaOH/l; 'ferric reagent', made by dissolving 200g of ammonium ferric sulphate in approx. 700ml of water and adding 93.4ml of conc. H_2SO_4 slowly. After cooling, the solution is made up to 1 litre with water.

The usual reaction mixture consists of 0.025M-sodium phosphate buffer containing 2mg of penicillin/ml; the reaction is started by adding an appropriate amount of enzyme. At suitable time-intervals, 2ml portions are pipetted into 6.5ml samples of a mixture (prepared that day) of hydroxylamine-alkaline buffer-water-ethanol (2:2:1:8, by vol.), mixed and allowed to stand for 7 min. A 2ml sample of 'ferric reagent' is then added, mixed thoroughly and the extinction at about 500nm read immediately in a colorimeter or spectrophotometer against a blank in which 2ml of buffer is treated in an identical fashion. For use with cephalosporins, a slight modification of this method is necessary; due to the instability of the hydroxamate formed with cephalosporins, the ferric reagent must be added after 3min (Hamilton-Miller, Smith & Knox, 1965; Hamilton-Miller, 1967). The unit of β -lactamase activity is that recommended by the International Commission on Enzymes (Thompson, 1962), i.e. that amount of enzyme which will catalyse the hydrolysis of one μ mol of benzylpenicillin/min at 30°C in 0.025M-phosphate buffer, pH 7.4.

(b) Iodometric (quantitative). For determination of K_m values, and in some other instances where a more sensitive assay was required, the micro-iodometric assay of Novick (1962) was used. This method, based on the iodometric assay described by Perret (1954), depends on the reduction of iodine, and hence the decolorization of the starch-iodine complex, by penicilloic acid (the product of the reaction) but not by penicillin. This was carried out as follows.

The buffer used was 0.05M-sodium phosphate, pH 7.4. A 2% (w/v) starch solution was made by suspending soluble starch (BDH) in buffer, heating to boiling with constant swirling, and then boiling gently for 5 min. The iodine solution, in 3.2M-KI, contained 10.15g of iodine/litre. The starch-iodine reagent was made by adding 0.15ml of iodine solution to 90ml of buffer, then adding slowly, with constant swirling, 10ml of starch solution. This reagent, and the starch solution, were prepared fresh daily.

Into each of two glass 1cm spectrophotometer cells were pipetted buffer (sufficient to give a final volume of 3.2ml), 0.2ml of starch solution, 1.0ml of starch-iodine reagent, and the required amount of penicillin solution in buffer, prepared freshly. (For measurement of enzyme activity, e.g. in column effluents, 0.1ml of a 2mg/ml solution of benzylpenicillin or oxacillin was used.) The

reaction was started by adding an appropriate amount of enzyme solution to the test cell, and the course of the reaction was followed by measuring the blank cell against the test cell at 620nm. Conditions were chosen such that the blank rate was not significant compared with the test rate.

(c) Iodometric (qualitative). For qualitative location of the enzyme in column effluents a spot-test modification of the iodometric assay was used.

Starch-iodine reagent consisted of 50ml of 1% soluble starch in 0.05M-sodium phosphate buffer, pH 7.4, +1 ml of iodine (10.15g/l in 3.2M-KI).

Penicillin solution (benzylpenicillin or oxacillin) was freshly made at 2mg/ml in 0.05M-sodium phosphate buffer, pH 7.4.

One drop of the sample was mixed with one drop of penicillin solution and left for 5 min; one drop of starch-iodine reagent was then added. Disappearance of the blue colour within 30 min indicates the presence of β -lactamase.

Protein concentration. This was measured by the spectrophotometric method of Waddell (1956) with bovine serum albumin as standard. Occasional checks were done by using the method of Lowry, Rosebrough, Farr & Randall (1951), as modified by Eggstein & Kreutz (1955). Column effluents were monitored at 278nm, by using a LKB Uvicord. Other methods are described in the appropriate Results section.

RESULTS

Purification of R-1818 β -lactamase from *E. coli*. The strain used for this was laboratory number 210.

Step (i). DM medium (Davis & Mingioli, 1950) (18 litres), supplemented (Smith, 1969) with tryptophan, histidine, proline and methionine was inoculated with a 6h starter culture. After shaking for 18h at room temperature (20–25°C), the culture was harvested in a Griffin-Christ Junior 1500 continuous-flow centrifuge and the pellet was washed with 400ml of buffer A. The yield of wet packed cells was normally about 20g.

The cells were resuspended in 80ml of buffer A, and divided into three portions; each was treated for two 5 min periods in an MSE 60W ultrasonicator, the vessel being cooled by immersion in a bath of ice-water. The three portions were combined and centrifuged at 27000g for 60min at 4°C. The pellet was discarded and the supernatant was termed the crude fraction.

All subsequent steps were performed at 4°C.

Step (ii). To the crude preparation was added 0.4vol. of a 2% solution of protamine sulphate in buffer A. After being allowed to stand for at least 30min, the precipitate was centrifuged off (5000g for 20min) and discarded. Solid ammonium sulphate was added to the supernatant to bring it to 25% saturation. The precipitate was discarded, and the supernatant was brought to 50% saturation with ammonium sulphate. The precipitate was collected by centrifugation and redissolved in 9 ml

of buffer A. During large-scale preparations, the extract was stored at this stage, and a number of such preparations were pooled.

Step (iii). The pooled ammonium sulphate precipitates were then dialysed overnight against 5 litres of 10mM-sodium phosphate buffer, pH 8.0, and applied to a column (60cm $\times$ 4cm²) of DEAE-cellulose (prepared as recommended by the manufacturer and equilibrated with 10mM-sodium phosphate buffer, pH 8.0). The β -lactamase was eluted with the same buffer, and the enzyme-containing fractions were pooled and dialysed overnight against 5 litres of 10mM-tris-HCl buffer, pH 8.9.

Step (iv). The contents of the dialysis tubing were then applied to a column (60 $\times$ 4cm²) of DEAE-cellulose equilibrated with 10mM-tris-HCl buffer, pH 8.9, and washed on with 50 ml of the same buffer. A linear gradient (1 litre) of 0–0.5M-sodium chloride in the same buffer was then applied; the enzyme was eluted at a concentration of sodium chloride of about 0.2M.

Step (v). The fractions containing β -lactamase were pooled, and solid ammonium sulphate was added to 60% saturation. The precipitate was collected by centrifugation and redissolved in 2ml of buffer A. This was then applied to a column (60cm $\times$ 4cm²) of Sephadex G-100 which was developed with buffer A by upward flow. The enzyme activity appeared at about 130 ml coincident with a single symmetrical protein peak (see Fig. 1). This indicates that the preparation is nearly homogeneous at this stage.

Step (vi). The pooled β -lactamase-containing fractions from the Sephadex column eluate were brought to 60% of saturation with solid ammonium sulphate. The precipitate was collected by centrifugation and redissolved in 2ml of buffer A.

Fig. 1. Purification of β -lactamase from *E. coli* J6-2 (R-1818) on a column (60cm $\times$ 4cm²) of Sephadex G-100. The buffer used was 0.025M-sodium phosphate, pH 7.4, and 3 ml fractions were collected. —, Percentage transmission at 278 nm; ○—○, β -lactamase (units/ml.)

The results of a typical purification using this procedure are shown in Table 1; about 9% of the original enzyme activity was recovered in the purified preparation.

Purification of R-1818 β -lactamase from P. mirabilis. The strain used for this was laboratory number 161.

The purification procedure was identical with that described above, except that the bacteria were grown in Oxoid nutrient broth no. 2. The results are given in Table 2; about 6% of the original enzyme activity was recovered in step (vi). The amount of protein obtained was too small to enable any assessment of purity to be made. However, it seems likely from the variation in specific activity across the enzyme peak obtained in step (v) that a substantial amount of impurity is still present in the preparation. It has not proved possible to purify completely this enzyme because the volume of initial culture needed would be greater than our resources would permit.

When the R-factor β -lactamase from *E. coli* was purified to the stage of apparent homogeneity, its specific activity was 143.2 units/mg of protein (Table 1). If the much lower activity (one-thirtieth) of R-factor specific β -lactamase in the *Proteus* preparation is solely due to enzyme modification, it follows that this protein would be pure when its specific activity was about one-thirtieth that of the pure *E. coli* enzyme, i.e. about 4.8 units/mg of protein. In fact, we were able to purify the *Proteus* enzyme until its specific activity was 31.3 units/mg of protein (Table 2). Even so, this enzyme was still impure.

Thus we can conclude that enzyme modification cannot solely account for the decreased activity of β -lactamase in *P. mirabilis* (R-1818). Indeed, since enzyme modification is usually reflected by alterations in enzymic properties and no differences of this nature were detected, it is likely that little, if any modification has occurred. Hence some mechanism must decrease the number of R-factor specific enzyme molecules produced by *P. mirabilis*.

Polyacrylamide-gel electrophoresis. This was done by the method of Ornstein and Davis (Ornstein, 1964; Davis, 1964) with a modified reservoir buffer at pH 8.6. Duplicate gels were run for each sample. One gel was stained for protein with 1% Amido Black in 7% (v/v) acetic acid; the other gel was used for location of the β -lactamase activity. This was done by slicing the gel transversely into 2mm discs, adding 1ml of buffer A to each and incubating overnight at 4°C. The supernatant was then assayed by the micro-iodometric method. The *E. coli* (R-1818) β -lactamase purified to step (v) showed only very minor contaminating bands (Fig. 2); the protein band corresponding to enzyme activity was rather broad.

Table 1. *Purification of R-1818 β -lactamase from E. coli J6-2 (R-1818) strain 210*

The procedure is described in detail in the text. These are pooled results from four preparations (total culture volume, 72 l) which were combined after step (ii). One unit of enzyme hydrolyses 1 μ mol of benzylpenicillin/min.

Step	Volume (ml)	Total protein (mg)	Total enzyme activity (enzyme units)	Specific activity (units/mg of protein)	Purification
(i) Crude enzyme	360	13460	6190	0.46	1
(ii) Ammonium sulphate precipitate (50% saturation)	46	2320	4070	1.76	4
(iii) First DEAE-cellulose column	80	72.5	1649	22.7	49
(iv) Second DEAE-cellulose column	50	19.9	760	38.2	83
(v) Sephadex G-100	28	6.0	410	68.3	148
(vi) Ammonium sulphate precipitate (60% saturation)	3.5	4.0	577	143.2	312

Table 2. *Purification of R-1818 β -lactamase from P. mirabilis F67 (R-1818) strain 161*

The procedure is described in detail in the text. These are pooled results from four preparations (total culture volume, 72 l) which were combined after step (ii). One unit of enzyme hydrolyses 1 μ mol of benzylpenicillin/min.

Step	Volume (ml)	Total protein (mg)	Total enzyme activity (enzyme units)	Specific activity (units/mg of protein)	Purification
(i) Crude enzyme	365	12400	169	0.0136	1
(ii) Ammonium sulphate precipitate (50% saturation)	48	4000	146	0.0365	3
(iii) First DEAE-cellulose	50	95.5	82.9	0.868	64
(iv) Second DEAE-cellulose	60	13.3	39.0	2.93	216
(v) Sephadex G-100	36	0.91	9.9	10.89	800
(vi) Ammonium sulphate precipitate (60% saturation)	3.5	0.36	10.9	31.30	2300

Substrate specificity. Datta & Kontomichalou (1965) measured the activity of a crude preparation of β -lactamase from *E. coli* K12 (R-1818) against a range of substrates. This has been extended to compare the substrate 'profiles' of crude and purified preparations of both *E. coli* J6-2 (R-1818) and *P. mirabilis* F67 (R-1818) β -lactamases by using the hydroxylamine assay, the reaction being started by addition of cold enzyme to the pre-warmed assay mixture (Smith, 1969). All substrates were used at 2mg/ml, with the exception of 6-aminopenicillanic acid, which was used at 1mg/ml. Except in the case of cloxacillin, the observed rates of hydrolysis were constant during the reaction, which involved a decrease in substrate concentration of at least 15% (usually much more); with cloxacillin as substrate, the rate of hydrolysis decreased rapidly during the course of reaction. The most likely explanation for this is that even at 2mg/ml the enzyme is not saturated (see 'Determination of K_m values' below). It was not possible to determine accurately the relative activities against the various substrates by using the same enzyme concentration. The rates of hydrolysis of propicillin, phenethicillin, oxacillin and cloxacillin were

therefore measured by using an enzyme concentration one-quarter that used for the other substrates; the rates were related to the rate of hydrolysis of benzylpenicillin by the same enzyme concentration. The results are shown in Table 3 together with, for the sake of comparison, the results obtained by Datta & Kontomichalou (1965). It can be seen that there are some significant discrepancies between the different assay conditions. Datta & Kontomichalou (1965) obtained their results by using an iodometric assay method at an unspecified temperature. It may be relevant that the R-1818 β -lactamase, in the absence of substrate and at the low concentrations used for assay, has been shown to be rapidly inactivated at temperatures greater than 28°C (Smith, 1969). The rate of inactivation is inversely dependent on enzyme concentration, so that, unless precautions similar to those used here are taken, one will tend to underestimate the activity against the 'better' substrates, where the enzyme concentration is lower.

The results obtained here show no significant difference in the β -lactamase specified by the R-1818 R-factor, whether harboured by *P. mirabilis* or *E. coli*. In addition, there is no significant difference

in the substrate specificities of the crude preparation and the purified enzyme in either case, which is consistent with the conclusion that the activity of the crude preparation is due entirely to one enzyme species.

Effect of pH on enzyme activity. The following buffers were used. McIlvaine's phosphate-citrate (one-quarter strength); 0.025 M-sodium phosphate; 0.05 M-sodium borate; 0.05 M-tris-HCl. R-1818 β -lactamase is stable for at least 7 weeks at 4°C in these buffers at the pH values used here (Dale, 1970).

The enzyme sample used was a small volume (less than 0.5% of the total assay volume) of crude preparation. To lower the risk of enzyme inactivation, the cold enzyme was the last component to be added to the pre-warmed assay mixture, and the

period of the assay was kept as short as possible. Controls were run for each buffer in the absence of enzyme to measure any spontaneous hydrolysis that might occur; the rate measured in the presence of enzyme was then corrected for this spontaneous rate. The curve obtained with McIlvaine's, phosphate and borate buffers for the *E. coli* (R-1818) enzyme acting on oxacillin is shown in Fig. 3. Similar curves are obtained with benzylpenicillin as substrate and with *P. mirabilis* (R-1818) β -lactamase against either substrate. The optimum pH was 7.2–7.4, with the 95% limits of activity being 7.0–7.6. R-1818 β -lactamase is unusual among Gram-negative β -lactamase enzymes in having a sharp peak, but is similar in this respect, as in several others, to the β -lactamase specified by the R-factor GN238 (Yamagishi, O'Hara, Sawai & Mitsuhashi, 1969). For a further discussion on the relationship between these two enzymes see Dale (1971).

The rate of spontaneous hydrolysis of benzylpenicillin or oxacillin in McIlvaine's, phosphate and borate buffers below pH 9 was not high enough to affect seriously the accuracy of the determination of the corrected enzyme rate. On the other hand, in the presence of tris-HCl buffer, both penicillins were rapidly destroyed, especially at pH values greater than 8; the rate was significant even at pH 7 (see also Schwartz, 1969). In addition, R-1818 β -lactamase is inhibited by Cl^- (Dale, 1971; see also Yamagishi *et al.* 1969); because of these two effects, tris-HCl buffer cannot be used for assaying this enzyme.

Determination of K_m values. Initial rates of reaction, at a minimum of nine different substrate concentrations, were measured by the micro-iodometric method at 20°C. The measured rates were corrected for the non-specific decolorization of the starch-iodine mixture by the proteins in the sample (i.e. the rate at zero substrate concentration).

K_m values were obtained by using the double-reciprocal plot (Lineweaver & Burk, 1934). The results (Table 4) show that the affinity of the R-1818 β -lactamase for a range of substrates is the same (within the limits of accuracy of the experiment) whether the host is *E. coli* or *P. mirabilis*.

Molecular weight. This was determined as described by Andrews (1964, 1965) by gel filtration on a column (60 cm $\times$ 4 cm²; Wright Scientific) of Sephadex G-100, by using upward flow; the buffer was 0.05 M-tris-HCl, pH 7.5, +0.1 M-KCl. All runs were done at room temperature (20–25°C). The fraction volume was 2 ml. The elution of protein was followed by measuring the extinction at 230 or 280 nm, Blue Dextran at 625 nm and cytochrome *c* at 412 nm; β -lactamase elution was followed by the micro-iodometric assay. The usual internal markers were bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c*. These were mixed,

Fig. 2. Polyacrylamide-gel electrophoresis of purified β -lactamase. Acrylamide gels (7.5%) were prepared and run as described by Ornstein (1964) and Davis (1964) with the reservoir buffer adjusted to pH 8.6. A Shandon analytical disc-electrophoresis apparatus was used, with tubes 75 mm long $\times$ 5 mm int. diam. About 30 μ g of R-1818 β -lactamase from *E. coli*, purified to step (v), was used in each of two tubes. Tube 1(a) was stained for protein with Amido Black; tube 2(b) was sliced and assayed for β -lactamase activity as described in the text.

Table 3. *Substrate profile of R-1818 β -lactamase*

Relative rates, in molar terms, measured by the hydroxylamine assay as described in the text. The rate against benzylpenicillin is taken as 100. The rate for cloxacillin is the initial rate (see the text for details). —, Not determined.

	<i>E. coli</i> J6-2(R-1818)		<i>P. mirabilis</i> F67(R-1818)		Average value	<i>E. coli</i> K12(R-1818)*
	Crude	Purified	Crude	Purified		
Benzylpenicillin	100	100	100	100	100	100
Ampicillin	188	206	156	152	175	91
Oxacillin	649	771	577	673	667	—
Cloxacillin	200	106	245	253	201	126
Penicillin V	180	161	232	178	188	92
Phenethicillin	342	477	350	379	387	180
Propicillin	545	633	480	571	557	240
Methicillin	17	—	39	—	28	21
6-Aminopenicillanic acid	53	84	71	64	68	73
Quinacillin	13	—	34	—	23	—
Carbenicillin	15	—	46	—	30	—
Cephaloridine	36	45	50	40	43	86
Cephalosporin C	16	—	43	—	29	—
Cephalothin	14	—	34	—	24	46

* Results taken from Datta & Kontomichalou (1965).

Fig. 3. Effect of pH on R-1818 β -lactamase activity. A crude preparation from *E. coli* (R-1818) was used with oxacillin as substrate. The buffers used were: ▲, McIlvaine's phosphate-citrate; ●, 0.025 M-sodium phosphate; ■, 0.05 M-borate.

before being applied to the column, with approx. 2 units of crude β -lactamase [with *E. coli* (R-1818)] or about 0.5 enzyme unit for *P. mirabilis* (R-1818).

By using this method, the molecular weight of R-1818 β -lactamase from *E. coli* J6-2 (R-1818) was found to be 44100; with *P. mirabilis* F67 as the

host, the molecular weight was 43900. In other words, within the limitations of the method, there is no difference in the molecular weights of the β -lactamase enzymes obtained from these two strains. When further results from other host bacteria are taken into account (Dale, 1971), the molecular weight of R-1818 β -lactamase is estimated as 44600 ± 2600 .

DISCUSSION

Molecular activity. From the value of 143 units/mg of purified R-1818 β -lactamase (Table 1), and taking the molecular weight as 44600 (Dale, 1971), the molecular activity (or 'turnover number') can be calculated to be 6.4×10^3 units/ μ mol of enzyme, i.e. 6.4×10^3 molecules of benzylpenicillin are hydrolysed/min per molecule of enzyme. This value is within the range of molecular activities determined for a number of β -lactamases (summarized by Citri & Pollock, 1966); this ranges from 2.76×10^3 for *Staphylococcus aureus* type B β -lactamase to 1.6×10^5 for the enzyme from *Bacillus cereus* 569. *E. coli* (R-TEM) β -lactamase has a molecular activity of 1.9×10^4 (Datta & Richmond, 1966).

R-factor gene expression. The results presented here support the view that R-1818 specifies the same β -lactamase whether harboured by *E. coli* J6-2 or by *P. mirabilis* F67, although the specific activity of crude extracts of the latter is only 6% of that of crude extracts of *E. coli* (R-1818) (Smith, 1969). Smith (1969) showed that there was a simple addi-

Table 4. K_m values (μM) of R-1818 β -lactamase

These were determined as described in the text with purified enzyme.

	<i>E. coli</i> J6-2 (R-1818)	<i>P. mirabilis</i> F67 (R-1818)
Benzylpenicillin	4.2	3.8
Ampicillin	6.8	5.9
Oxacillin	88.8	92.5
Penicillin V	14.3	16.0
Phenethicillin	11.8	19.3
Propicillin	21.8	17.9
6-Aminopenicillanic acid	80.2	42.7
Cephaloridine	75.4	42.1

tive effect in mixtures of crude enzyme preparations from *E. coli* (R-1818) and *P. mirabilis* (R-1818); this excludes the possibility of an inhibitor of R-1818 β -lactamase being present in *P. mirabilis* cell sap, or of a stimulatory factor in *E. coli*. In the same paper he reports that if the R-factor is transferred from the *Proteus* strain to *E. coli*, the β -lactamase activity is similar to that in the original *E. coli* (R-1818), which indicates that no inheritable modification of the R-factor occurs while in the *Proteus*. The most probable reason for the decreased activity of *Proteus* extracts is therefore a decrease in the amount of enzyme synthesized per cell.

Falkow, Wohlheiter, Citarella & Baron (1964) and Colby & Hu (1968a) found anomalous regulation of synthesis of β -galactosidase by *P. mirabilis* carrying an F-lac episome transferred from *E. coli*. In this case also, the enzyme produced was apparently identical with that specified by the episome in *E. coli* (Falkow *et al.* 1964; Colby & Hu, 1968b), but the fully induced activity (enzyme units/10⁹ bacteria) of β -galactosidase in *P. mirabilis* F-lac was of the order of 6% of the induced activity in *E. coli* lac⁻/F-lac (Colby & Hu, 1968a). This is strikingly similar to the values of 6 and 7% obtained by comparing the β -lactamase activities in *P. mirabilis* carrying the 1818 or 7268 R-factors, respectively, with the activities in *E. coli* carrying the relevant R-factors (Smith, 1969). On the other hand, *P. mirabilis* (R-TEM) produces only 0.9% of the β -lactamase activity of *E. coli* (R-TEM) (Smith, 1969); however, subsequent work has shown that if log-phase cells, grown at 37°C, or cells from an overnight 30°C culture, are used, the activity is indeed similar at 6% of the activity of *E. coli* (R-TEM) (J. T. Smith & S. R. Hesslewood, unpublished work).

This suggests that the decreased expression of R-factors and the F-lac episome in *Proteus* may be due to a similar cause. Colby & Hu (1968a) suggest that the conformation of the repressor molecule is altered by the different cytoplasmic environment in *Proteus*; a similar explanation for the regulation of

R-factor β -lactamase seems unlikely since all attempts to induce R-factor β -lactamases have so far failed.

One alternative explanation is that 'foreign' DNA is inefficiently expressed by *P. mirabilis*. Several workers have shown, by using *E. coli* RNA polymerase, that the amount and type of RNA synthesized *in vitro* varies to some extent according to the DNA template and RNA polymerase used (Furth, Hurwitz & Anders, 1962; Stevens & Henry, 1964; Burgess, Travers, Dunn & Bautz, 1969). *E. coli* RNA polymerase can be separated by chromatography on phosphocellulose into two components; the 'core' polymerase and the σ factor (Burgess *et al.* 1969; Darlix, Sentenac, Ruet & Fromageot, 1969). The σ factor is necessary for accurate initiation of transcription (Travers & Burgess, 1969; Summers & Siegel, 1969). It is possible that *P. mirabilis* contains a σ factor that does not recognize the initiation sites on 'foreign' DNA, as represented by the F-lac episome or the β -lactamase genes on the R-factors. However, there are alternative hypotheses (e.g. specificity may reside at the translational level) that cannot be excluded at present.

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