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Distribution of β-Lactamases A and B in some Groups of *Yersinia enterocolitica* and their Role in Resistance

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SUMMARY

Yersinia enterocolitica W222 (serological group 3) synthesized two different intracellular β-lactamases, called A and B. Enzyme B was more sensitive than A to inhibition by cloxacillin. The minimum inhibitory concentrations of various β-lactam antibiotics for strains of *Y. enterocolitica* of groups 3 and 9 and the effect of cloxacillin on these concentrations suggested differential roles for β-lactamases of types A and B in penicillin and cephalosporin resistance. Type B enzymes protected *Y. enterocolitica* against cephalothin and cephalosporin C, whereas type A enzymes protected very efficiently against carbenicillin. Protection against other β-lactam antibiotics was exerted by both enzymes. However, while both enzymes readily hydrolysed cephaloridine and showed no crypticity with this substrate, they only conferred a very weak protection against it. This may be because cephaloridine reached its target quickly, before it was degraded. The resistance of strains of *Y. enterocolitica* from groups 1, 2 and 16 was also explicable in terms of a two-enzyme system, whereas strains belonging to group 5b produced only a type B lactamase and were sensitive to carbenicillin.

INTRODUCTION

Antibiotics of the β-lactam group are relatively ineffective against *Yersinia enterocolitica* (Nilhen, 1967, 1969; Wauters, 1970; Zen-Yoji & Maruyama, 1972) and strains of this species are β-lactamase producers (Cornelis, Wauters & Vanderhaeghe, 1973; Mishankin, Ryzhko & Grigorian, 1973). While all the different serological groups (Winblad, 1967, 1968; Wauters, Le Minor & Chalon, 1971; Wauters *et al.* 1972) of *Y. enterocolitica* are β-lactamase producers, they differ from one another in the pattern of their sensitivities to β-lactam antibiotics (Cornelis *et al.* 1973).

An attempt to purify the β-lactamase from strain W222 (chosen at random among group 3 strains) revealed that this strain produced two different β-lactamases which have been called A and B. Three other strains from group 3 and two strains from group 9 also had two lactamases. In contrast, strain H66, an unusual carbenicillin-sensitive strain, synthesized only one lactamase which was very similar to B (Cornelis & Abraham, 1975). Among the characteristics which distinguished β-lactamases A and B were their sensitivity to inhibitors: A was more sensitive to thiol reagents, and B to inhibition by cloxacillin (Cornelis & Abraham, 1975).

My aim was to assess the respective roles of these two β-lactamases in the resistance of *Y. enterocolitica* to β-lactam antibiotics. Three methods were used. One involved the determination of the minimum inhibitory concentration (m.i.c.) of each of a number of β-lactam antibiotics for strains from different groups, including strain H66 and a mutant specifically impaired in ability to produce one of the two lactamases. A second involved a

study of the selective effect of cloxacillin and other B-enzyme inhibitors on the pattern of m.i.c. values. The third method determined the response to different β-lactam antibiotics of crude extracts of strains from the different groups, to correlate the enzymic properties of these extracts with the m.i.c. data. The present work deals with the serological groups 3, 9 and 5b, mainly encountered in man, and 1, 2 and 16 usually encountered in animals (Nilhen, 1969; Wauters, 1970; Zen-Yoji *et al.* 1973; Aldova & Lim, 1974). Groups 9 and 5b show the same biochemical behaviour and are therefore classified in the same biotype; the remaining groups belong to different biotypes (Wauters, 1970).

METHODS

Strains. The strains studied were the same as those used by Cornelis *et al.* (1973) except that V.2.73, an R factor-carrying strain, was omitted and sixteen strains of serotype 5b were added. The latter strains were also members of Professor G. Wauters' (Louvain) collection. They include the two sole indole(−) 5b strains of this collection (YE850 isolated by Weaver, and TI10 isolated by Toma).

Minimum inhibitory concentrations. These values were determined by the agar dilution method (Ericsson & Sherris, 1971) using a multipoint inoculator (Denley, Bolney, Sussex). At each step in a series of dilutions, the concentration of antibiotic was increased by a factor of two. The medium was Mueller-Hinton agar (Difco) supplemented with 5 % (v/v) defibrinated horse blood. The plates were incubated for 24 h at 29 °C.

Isolation of a carbenicillin-sensitive mutant. A broth culture (5 ml) of strain W238 in the exponential phase of growth was centrifuged and the pellet resuspended in 5 ml of 0·066 M-phosphate buffer pH 6·0 containing the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; 30 μg/ml; Fluka A.G., Buchs, Switzerland). This suspension was incubated for 15 min at 37 °C, then centrifuged and washed twice in 5 ml buffer; 0·1 ml of the washed bacterial suspension was transferred to 5 ml broth and incubated overnight. The mutagenized culture was then diluted and plated on tryptic soy agar (TSA; Difco). Plates bearing separated colonies were replicated on TSA containing carbenicillin (100 μg/ml) or cephalosporin C (200 μg/ml). Of 1600 colonies replicated, ten did not grow, or grew poorly, on carbenicillin. Of these, only one (W23801) was a carbenicillin-sensitive mutant.

Detection of synergy by a diffusion method. The bacteria were inoculated on TSA or Mueller–Hinton agar in order to yield a dense but not completely confluent growth. Samples (50 μl) of the antibiotic solutions to be tested were placed in 7 mm diameter holes punched in the agar at a distance of 17 mm (centre to centre) from each other. The concentrations were 2 and 4 mg/ml with cephalosporin C, 0·25, 0·5 and 1·0 mg/ml with cephalothin, and 0·062 and 0·125 mg/ml with cephaloridine. The methicillin concentrations were 1·0 and 2·0 mg/ml for strain W222 and 0·25 and 0·5 mg/ml for strain H66. The carbenicillin concentrations were 0·5, 1·0 and 2·0 mg/ml.

Growth. The cultures were grown as previously described (Cornelis & Abraham, 1975), except that normal 500 ml conical flasks shaken at 250 rev/min were used and the temperature was 29 instead of 27 °C. Growth was followed with a Klett–Summerson colorimeter (New York, U.S.A.) using filter W66. I attempted to induce the formation of a β-lactamase by adding 6-aminopenicillanic acid after 4 h growth. The bacteria were harvested after 7 h growth.

Preparation of crude extracts. This was done as described by Cornelis & Abraham (1975), except that the thawed bacteria were mixed with water instead of phosphate buffer. The ultrasonic disintegrator was a Branson (Danbury, Connecticut, U.S.A.) sonifier (80 W,

20 kHz) equipped with a microtip. The time of centrifugation (100000 *g*) was decreased to 1 h. Crude extracts to be kept for some days were immediately dialysed against phosphate buffer (0·025 M, pH 7·3).

Starch gel electrophoresis was performed according to Smithies (1955). The gels were made up of 15 g hydrolysed starch (Connaught, Toronto, Canada) in 100 ml of 0·03 M-borate buffer, pH 7·5 or 8·0. The electrolyte (400 ml in each compartment) was the same buffer at 0·3 M. Samples (5 μl) were absorbed on small strips (10 × 1 mm) of Whatman No. 3 paper. Runs were made for 2·5 h under constant voltage (300 V) in a Shandon U77 apparatus. The enzymes were revealed by the method of Jack & Richmond (1970): the gels were sprayed with a solution containing benzylpenicillin (10 mg/ml), 0·016 M-I_2, 0·12 M-KI and 0·066 M-phosphate buffer, pH 6·5. This coloured the gels blue and after a few minutes incubation at 37 °C, β-lactamases appeared as colourless zones. Samples of enzymes A and B from W222 (group 3) were used as references (Cornelis & Abraham, 1975). A crude extract of *Y. pseudotuberculosis* 360 was used as a negative control of the lactamase staining. Enzyme A remained at the origin, rather on the cathodic side of the strip, while enzyme B moved towards the anode.

Assay of β-lactamases. β-Lactamase activity is expressed as μmol substrate hydrolysed/min/ml enzyme solution, at 30 °C and pH 6·5. The rate of hydrolysis was determined by the alkalimetric method as previously described (Cornelis & Abraham, 1975). The apparatus (Radiometer, Copenhagen, Denmark) comprised a pH meter 61, a TT 60 titrator, an ABU 12 autoburette and a Rec 61 servograph. The alkali was 50 mM-NaOH instead of 40 mM-NaOH.

Small-scale preparation of enzyme B from strain W238. After 48 h dialysis, 0·5 ml crude extract from strain W238 was applied to a small column (17·5 × 0·5 cm) containing 3 ml Sephadex CM-50 (Pharmacia) equilibrated with 0·025 M-phosphate buffer pH 7·3. Enzyme B passes straight through the column while enzyme A is retained (Cornelis & Abraham, 1975). The first active fraction was recovered and checked by starch gel electrophoresis for lack of enzyme A.

Substrates and inhibitors. Benzylpenicillin was from Ludeco, Brussels, Belgium; cephalosporin C and cephaloridine were from Glaxo Research Ltd, Greenford, Middlesex; cephalothin, cephalexin and cefamandole were from Eli Lilly & Co., Indianapolis, U.S.A.; 6-aminopenicillanic acid (6APA), cloxacillin, methicillin, carbenicillin and ampicillin were from Beecham Research Laboratories, Brockham Park, Surrey; oxacillin was from Bristol Benelux, Brussels, Belgium; cefoxitin was from Merck Sharp and Dohme, Rahway, New Jersey, U.S.A.

RESULTS

Minimum inhibitory concentrations

The m.i.c. values of some penicillins and cephalosporins were determined for all the strains mentioned in Methods. As observed before (Cornelis *et al.* 1973), the m.i.c. values were generally very similar for strains within each serological group. Table 1 gives only the most frequently observed value, with each antibiotic, for strains of each group. The individual and experimental variations were normally one dilution and very exceptionally two dilutions (i.e. a factor of two or four between the m.i.c. values). However, within group 2, the individual variation in the m.i.c. for cephalosporin C was unusually high and the values for the two extreme strains are given in Table 1. The m.i.c. values for *Y. pseudotuberculosis*, which does not produce a detectable β-lactamase (Cornelis *et al.* 1973; Mishankin *et al.* 1973), have been determined for comparison. As Cornelis *et al.* (1973) noted, group 5b

Table 1. *Mean minimum inhibitory concentrations of β-lactam antibiotics*

The values are the means obtained with all the strains mentioned in Methods.

Organism	Minimum inhibitory concentration (μg/ml)												
	6APA	Oxacillin	Methicillin	Cloxacillin	Carbenicillin	Cefamandole	Ampicillin	Benzylpenicillin	Cephaloridine	Cephalexin	Cefoxitin	Cephalothin	Cephalosporin C
Y. pseudotuberculosis	32	32	32	16	0·5	0·5	0·25	0·5	1	2	0·5	0·5	4
Y. enterocolitica group 1	128	128	512	128	512	4	64	128	16	32	64	256	1024
Y. enterocolitica group 2													
Strain A14700	128	64	256	64	256	1	32	32	4	8	4	32	512
Strain F96–1	64	32	256	32	128	2	32	16	2	8	1	16	16
Y. enterocolitica group 3,	128	128	512	64	512	4	64	128	8	32	4	128	512
Strain H66	32	32	32	32	0·5	0·5	8	32	4	8	4	32	512
Y. enterocolitica group 5b (except indole(−) strains)	64	64	128	64	2	2	32–64	256	32	64	64	512	1024
Y. enterocolitica group 9	128	64	256	64	256	2	64	128	32	64	64	512	1024
Strain W23801	32	—	16	—	2	0·25	2	8	2	—	8	32	1024
Y. enterocolitica group 16	64	128	256	128	256	2	128	256	16	64	32	256	1024

Table 2. *Inhibition of Y. enterocolitica β-lactamases by poorly hydrolysed β-lactam antibiotics*

The activity of enzyme A was determined with cephaloridine as a substrate. Cephalosporin C was the substrate used for enzyme B and for the crude extract of strain M771. The inhibitor concentration was 10^{-4} M. Preparations of enzyme A (W222) and B (W222) were described previously (Cornelis & Abraham, 1975).

	Inhibition (%)		
Inhibitors	β-Lactamase A (W222)	β-Lactamase B (W222)	Crude extract (M771)
Cloxacillin	28*	> 99*	> 99
Methicillin	64	> 99	NT
Carbenicillin	NT	> 99	> 98
Cefoxitin	22	93	> 99
Cefamandole	6	18	NT
Ampicillin	NT	64	77

NT = Not tested. * Data from Cornelis & Abraham (1975).

contrasts strikingly with the other groups in the m.i.c. values for carbenicillin. The indole(−) strains, however, appear as resistant to carbenicillin as strains belonging to other groups. Group 3 strains are highly resistant to carbenicillin and cephalosporin C, except strain H66 which is highly resistant to cephalosporin C but sensitive to carbenicillin. The strains from group 9 are also very resistant to carbenicillin and cephalosporin C. Strain W23801, a carbenicillin-sensitive mutant from strain W238, remained highly resistant to cephalosporin C and moderately resistant to cephalothin.

β-Lactamase inhibition in vitro by β-lactam antibiotics

Cloxacillin (10^{-4} M) completely inhibits the activity of enzyme B but only partially that of enzyme A (Cornelis & Abraham, 1975). Other β-lactam antibiotics which are poorly hydrolysed are also inhibitors of enzyme B (Table 2), among them carbenicillin, cefoxitin (a 7-methoxycephalosporin) and ampicillin. On the other hand, ampicillin and carbenicillin are substrates from enzyme A (Cornelis & Abraham, 1975), while cefoxitin, which is not hydrolysed by A, is only a poor inhibitor of this enzyme.

Synergy with different β-lactam antibiotics

Since enzyme B can be inhibited *in vitro* by certain β-lactam antibiotics which act as substrate analogues, synergy between these inhibitors and different β-lactam antibiotics seemed possible, as with some other β-lactamase-producing Gram-negative bacteria (Hamilton-Miller, Smith & Knox, 1964; Sabath & Abraham, 1964; Sutherland & Batchelor, 1964; Hamilton-Miller, 1971). Using a diffusion method, synergism was clear between methicillin and cephalosporin C, or cephalothin, with strains W222 and H66. Synergism was also evident from the considerable falls (3 to 5 dilutions) in the m.i.c. values for cephalosporin C against strains of all the groups when cloxacillin (10 or 20 μg/ml) was present. Since enzyme B is more sensitive than enzyme A to inhibition by cloxacillin, the degree of synergy between cloxacillin and various penicillins and cephalosporins was used to assess the respective roles of these two enzymes in resistance. For each determination of m.i.c. values, the strains were inoculated in parallel on solid medium with and without cloxacillin (20 μg/ml, 4×10^{-5} M). There was again a great uniformity within the groups and the results have been condensed (Table 3). The number of dilutions are recorded between the m.i.c.

Table 3. *Synergy between cloxacillin and other β-lactam antibiotics against Y. enterocolitica*

Minimum inhibitory concentrations were determined by the agar dilution method, in parallel, with and without cloxacillin incorporated into the medium. For each group, three to five strains were used. The first numbers (mean values) represent the number of dilutions between the m.i.c. of the antibiotic determined with and without cloxacillin, i.e. the difference between the $\log_2$ of these m.i.c. values. The numbers in parentheses, representing the differences between the $\log_2$ of the m.i.c. (without cloxacillin) for *Y. enterocolitica* and *Y. pseudotuberculosis* are given as an indication of the contribution of the β-lactamases to resistance. The cloxacillin concentration was 20 μg/ml except for strain H66 where it was 10 μg/ml (m.i.c. 32 μg/ml).

	Carbenicillin	Ampicillin	Benzyl-penicillin	Cefa-mandole	Cephal-oridine	Cefoxitin	Cephalothin	Cephalo-sporin C
Y. enterocolitica group 1	0 (10)	1 (8)	2 (8)	1 (3)	3 (4)	5 (7)	4 (9)	4 (8)
Y. enterocolitica group 3	0 (10)	1 (8)	2 (8)	1 (3)	2 (3)	2·5 (3)	3 (8)	3 (7)
Strain H66 (group 3, *penA*$^-$)	0·5 (0)	6 (5)	6 (6)	NT	2 (2)	NT	NT	6 (7)
Y. enterocolitica group 5b	0·5 (2)	6 (7·5)	6 (9)	1 (2)	5 (5)	5 (7)	7 (10)	5·5 (8)
Y. enterocolitica group 9	1 (9)	2 (8)	3·5 (8)	1 (2)	4 (5)	6 (7)	6 (10)	4·5 (8)
Y. enterocolitica group 16	0 (9)	2 (9)	NT	1 (2)	4 (4)	4 (6)	5 (9)	4 (8)

NT, not tested.

Table 4. *Synergy between carbenicillin and other β-lactam antibiotics for Y. enterocolitica*

Minimum inhibitory concentrations were determined by the agar dilution method, in the absence of carbenicillin and in its presence at various concentrations (20 to 100 μg/ml).

	Minimum inhibitory concentration (μg/ml)											
	Ampicillin				Cephalosporin C				Cephalothin			
Carbenicillin concn (μg/ml) ...	0	20	50	100	0	20	50	100	0	20	50	100
Group 1 (Ye128)	64	32	16	8	2048	256	32	4	256	32	8	8
Group 2 (AI4700)	32	16	16	16	512	128	64	32	32	16	8	8
Group 3 (W223, W224, W237, W239)	64	32	32	32	512	64–128	64	32–64	128	32	16	16
Group 9 (W228, W244, W410)	64	32	16–32	16	1024	512	256	128	512	32	16	16–8
Group 16 (AZ1523)	128	16	8	4	1024	512	128	32	256	16	8	4

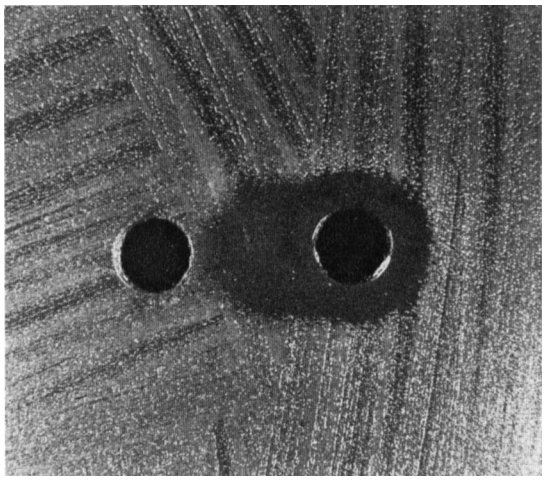

Fig. 1. Synergy between carbenicillin and cephalosporin C. The plate was seeded with *Y. enterocolitica* strain W239 (group 3). Left: carbenicillin (1 mg/ml); right: cephalosporin C (4 mg/ml). Each hole received 50 μl antibiotic solution.

values with and without cloxacillin, i.e. the differences between the $\log_2$ of the two values. Cloxacillin at 10 μg/ml gave results which were either identical or one unit lower.

For the groups 1, 3, 9 and 16, the effect of cloxacillin was far from uniform, even when only the antibiotics with very high m.i.c. values are compared. Synergy was very marked with cephalosporin C and cephalothin while it was not, or nearly not, observable with carbenicillin. Moreover, synergy was generally greater in group 9 than in group 3. By contrast, this differential synergy between cloxacillin and other β-lactam antibiotics was not observed for strain H66 and those of group 5b, if the results with carbenicillin (to which these strains are already fully sensitive in the absence of inhibitor) are neglected.

Since carbenicillin is a substrate for enzyme A but an inhibitor for enzyme B, synergy was looked for between carbenicillin (at 20, 50 and 100 μg/ml) and other β-lactam antibiotics, with the *Y. enterocolitica* groups highly resistant to carbenicillin. Synergy was found between

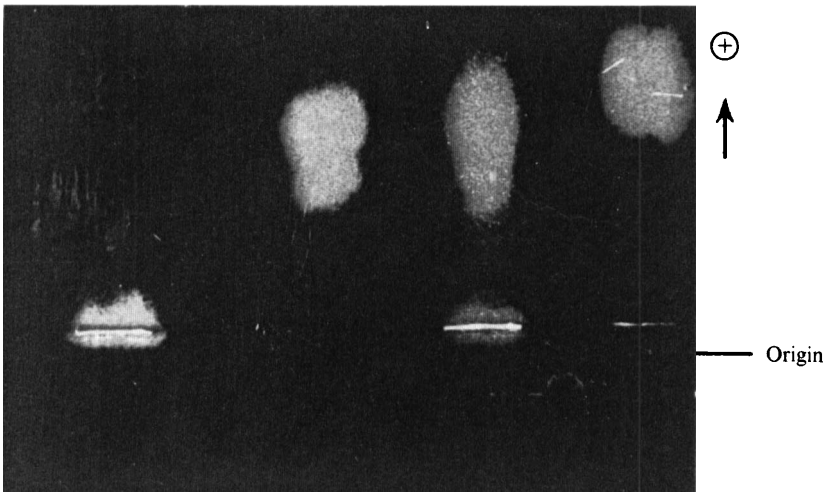

Fig. 2. Starch gel electrophoresis at pH 7·5 of enzymes A (I) and B (IV) from strain W222 (group 3) and crude extracts from strains M771 (group 5b, II) and W238 (group 9, III). The β-lactamases were stained with benzylpenicillin and iodine.

carbenicillin and the three other β-lactam antibiotics tested (Table 4). As when cloxacillin was inhibitor, the effect was greater for cephalothin and cephalosporin C than for ampicillin. For group 5b, carbenicillin was used at a concentration of 0·5 μg/ml (m.i.c. 2 μg/ml), but no synergistic effect could be detected at this concentration ($\simeq 10^{-6}$ M). Synergy between carbenicillin and cephalothin, or cephalosporin C, was also revealed by the diffusion technique with the group 3 strain W239 (Fig. 1). With those groups for which the m.i.c. of cefoxitin alone was 64 μg/ml, m.i.c. values were determined for cephalosporin C and cefoxitin (20 μg/ml) together. In contrast to the result with cloxacillin and cephalosporin C, only a slight synergy (1 dilution) could be observed.

β-Lactamase A and B content of crude extracts

In general, starch gel electrophoresis of crude extracts of strains F96-1, A14700 and W238 (groups 2 and 9) indicated that both enzymes A and B were present. Strain M771 (group 5b), on the other hand, exhibited only one spot which corresponded to B (Fig. 2). The B-like spot of strain W23801 corresponded with that of W238, but the A-like spot was weak compared with that of W238. The β-lactamase in the crude extract from strain YE110 (group 1) appeared to separate into two spots, but these were closer together than those of A and B: the enzyme with the greater mobility, which seemed to be present in the greater amount, migrated less far than B, while the other migrated slightly towards the anode. Strain AZ1523 (group 16) gave only a diffuse band of low mobility, with a slight constriction in the middle.

Comparison of the characteristics of the enzyme from strains W222, W238 *and* M771

A preparation of the B-like enzyme from strain W238 was obtained by small-scale chromatography on Sephadex CM-50. The substrate profile of this preparation was compared with the profile of B (W222) and the crude extracts from W238 and M771 (Table 5). This revealed that B (W222), B (W238) and M771 have similar profiles, but that B (W238)

Table 5. *Substrate profiles*

The rates of hydrolysis were determined with a pH stat, at 30 °C and pH 6·5, with substrate concentration of 2 mg/ml. The rates are relative to an arbitrary value of 100 for benzylpenicillin. The numbers in parentheses identify the strains from which the β-lactamase was extracted.

	Rates of hydrolysis (relative) by				
Substrate	Enzyme A (W222)*	Enzyme B (W222)*	Enzyme B (W238)†	Crude extract (strain M771, group 5b)	Crude extract (strain W238, group 9)
Cephalosporin C	35	605	450	324	101
Cephaloridine	300	277	221	190	214
Cephalothin	134	214	NT	86	NT
Benzylpenicillin	100	100	100	100	100
Ampicillin	77	< 1	NT	⩽ 2	NT
Carbenicillin	25	< 1	NT	< 1	NT

* Data from Cornelis & Abraham (1975).
† Prepared as described in text.
NT, Not tested.

Table 6. *Specific enzyme activities and inducibility*

For most strains, three inducer concentrations were used (500 μg/ml, 200 or 250 μg/ml, and 750 or 1000 μg/ml). For strains H66 and W23801, the range of inducer concentration was lower (100 μg/ml, 200 or 250 μg/ml, and 500 μg/ml).

	$10^2 \times$ enzyme activity (enzyme units/mg dry wt organisms) against:		
		Cephalosporin C	
Strain	Cephaloridine. Non-induced	Non-induced	Induced
YE110 (group 1)	10·0	5·0	7·8
AI4700 (group 2)	9·3	2·1	2·2
F96·1 (group 2)	7·7	1·2	1·0
W222 (group 3)	14·1	4·3	7·3
W239 (group 3)	13·2	3·2	4·3
H66 (group 3)	—	0·6	7·0
M771 (group 5b)	5·3	9·0	10·5
W238 (group 9)	11·8	6·2	10·8
W23801 (group 9)	5·9	6·4	7·3
AZ1523 (group 16)	4·8	2·2	2·6

—, Not tested in this experiment because this strain possesses only the inducible enzyme B.

and M771 are relatively more active against benzylpenicillin. The β-lactamase activity of M771 crude extract was strongly inhibited by cloxacillin, carbenicillin and cefoxitin (10^{-4} M) and also partially inhibited by 10^{-4} M-ampicillin (Table 2).

Specific activity and inducibility of β-lactamases of crude extracts

Since it may be assumed that the activity *in vitro* against cephaloridine is mainly due to enzyme A, while that against cephalosporin C is mainly due to B (see substrate profiles), the specific activities of the crude extracts from the different strains were determined with these two substrates. However, an earlier study of strains W222 and H66 (Cornelis & Abraham, 1975) had revealed that enzyme B was inducible. Consequently, a study was made of the ability of 6APA to act as an inducer with all the strains. The activity of strain

H66 against cephalosporin C was increased about ten times by addition of 6APA (200 μg/ml) (Table 6). For the strains W238 (group 9), W222 (group 3) and YE110 (group 1), only a slight increase in activity was observed with 6APA at 500 or 750 μg/ml (induction ratio 1·6 or 1·7) while there was no effect, or nearly none, with the other strains.

Crypticity

To examine the accessibility barrier in *Y. enterocolitica* towards some β-lactam antibiotics, the β-lactamase activity of intact bacteria from strain W239 (group 3) was compared with that of sonically disrupted bacteria, with these antibiotics as substrates. These two values were identical when cephaloridine was substrate. The activity of disrupted bacteria was 3 times higher with cephalosporin C, four times with cephalothin, seven times with cefamandole and 7·5 times with benzylpenicillin. With carbenicillin, the ratio was higher than 50.

DISCUSSION

Role of β-lactamases A and B in the resistance of Y. enterocolitica 3 and 9. The m.i.c. patterns of the strains of *Y. pseudotuberculosis* and the strains of *Y. enterocolitica*, including H66, suggest that resistance to carbenicillin is correlated with the presence of β-lactamase type A. On the other hand, resistance to cephalosporin C seems to be mainly associated with the presence of enzyme B. The m.i.c. pattern of the mutant W23801 (group 9) leads to the same conclusion. The crude extract of this mutant is specifically impaired in its ability to hydrolyse cephaloridine, in agreement with the diminution of the electrophoretic spot corresponding to type A enzyme.

The idea that resistance to cephalosporin C is mainly due to enzyme B, while the resistance to carbenicillin is mainly due to enzyme A, is also consistent with the results of the experiments on synergism. The B inhibitor (cloxacillin) is synergistic with cephalosporin C but not with carbenicillin. While hydrolysed by enzyme A, carbenicillin still appears to inhibit enzyme B, since strong synergy is shown by carbenicillin + cephalosporin C or carbenicillin + cephalothin. Bobrowski & Borowski (1971), who showed the inhibitory effect of carbenicillin on β-lactamases from *Pseudomonas aeruginosa* and Enterobacter, did not observe significant synergism between carbenicillin and cephaloridine, which was rapidly hydrolysed by these lactamases. They suggested that the absence of synergism could be explained by the high rate of penetration by cephaloridine through the surface layers of the organism and the existence of a permeability barrier to carbenicillin. In the present work, two β-lactam antibiotics were used (cephalosporin C and cephalothin) which showed some crypticity and which were readily hydrolysed by the β-lactamase concerned. Moreover, the resistance to carbenicillin due to enzyme A allowed the use of a relatively high concentration of this antibiotic in the synergy experiments. Assuming that the synergy between cloxacillin and other β-lactam antibiotics is an expression of the part played by enzyme B in the resistance to these antibiotics, an indication of the importance of B in resistance can be obtained from the intensity of the synergy. The differences between the resistance levels of H66 (B only) and the other group 3 strains (A + B) provides an indication of the role of the A-type β-lactamase.

A striking indication of the effect of the enzymes on resistance is provided by the activity of carbenicillin, which is only slightly hydrolysed by A and is a strong inhibitor of B. On the other hand, the m.i.c. values of cephaloridine are low in spite of the high rate of hydrolysis of this substrate by both enzymes. However, with cephaloridine relatively high K_m values could lower the effectiveness of the enzyme *in vivo*. The K_m value of A (W222) for

cephaloridine is 175 μM ($\simeq$ 70 μg/ml) while it is only 39 μM ($\simeq$ 14 μg/ml) for benzylpenicillin (Cornelis & Abraham, 1975). In addition to this, the 'crypticity factor' for cephaloridine is extremely low and, as shown by Hamilton-Miller (1963) and Richmond & Sykes (1973), the higher the crypticity factor for a substance, the higher tends to be the protection by a given β-lactamase.

Resistance pattern of group 5b. The sensitivity to carbenicillin and the uniform synergy between cloxacillin and other β-lactam antibiotics observed in group 5b can be explained by the lack of type A β-lactamase revealed by starch gel electrophoresis. The fact that the profile of the crude extract of strain M771 (group 5b) is similar to that of B (from strain W222), agrees with this conclusion. The resistance to ampicillin observed with the strains of group 5b remains unaccounted for in view of the type B β-lactamase profile. Moreover, the synergy with cloxacillin + ampicillin observed with these strains raises the question whether the B enzyme is actually involved in this resistance. The same kind of situation was, however, observed with *Escherichia coli*: some strains selected for ampicillin resistance were found to produce a β-lactamase with extremely low activity against ampicillin (Smith, 1963; Lindström, Boman & Steele, 1970). Similarly, Roupas & Pitton (1974), in a survey of ampicillin resistance in *E. coli*, found some ampicillin-resistant strains which synthesized a cloxacillin-sensitive β-lactamase with extremely low activity *in vitro* against ampicillin (profile E of these authors). A synergy cloxacillin + ampicillin provided evidence, also, for an effect of the lactamase in the ampicillin resistance.

Resistance patterns of groups 1, 2 *and* 16. With the strains belonging to group 2, electrophoresis of crude extracts showed the presence of a type A and a type B β-lactamase. The type A enzyme was still predominant, in agreement with the specific activities against cephalosporin C and the m.i.c. pattern. Strains from groups 1 and 16 are highly resistant to carbenicillin and there is synergy between cloxacillin, or carbenicillin, and other β-lactam antibiotics with these strains. This suggests strongly that the strains have a two-enzyme system, but no clear confirmation of the presence of two β-lactamases was obtained by gel electrophoresis. It is possible that, within these groups, the enzymes corresponding to β-lactamases A and B have similar electrophoretic mobilities under the conditions used.

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Corrigendum. In G. Cornelis & E. P. Abraham (1975), *Journal of General Microbiology* **87**, pp. 273–284, on p. 276, line 3 of Results: for 'about 7×10^{-1}' read 'about 7×10^{-2}'.

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