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R Factor-Mediated and Chromosomal Resistance to Ampicillin in *Escherichia coli*

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Sixty-four ampicillin-resistant strains of *Escherichia coli* were studied. Six characters were examined: (i) resistance to ampicillin, cephalothin, and carbenicillin, (ii) synergy between ampicillin and cloxacillin, (iii) level of β-lactamase activity after osmotic shock, (iv) transferability of ampicillin resistance, (v) immunological characterization of the enzyme, and (vi) determination of substrate profiles. One class of strains was found in which synthesis of β-lactamase is inferred to be plasmid mediated; these strains are highly resistant to ampicillin and carbenicillin, sensitive to cephalothin, do not show synergism between ampicillin and cloxacillin, and reveal a high enzymatic activity after osmotic shock. A second class is formed by strains for which β-lactamase synthesis is inferred to be chromosomal; these strains present a low resistance level to ampicillin, are sensitive to carbenicillin and resistant to cephalothin, show a synergism between ampicillin and cloxacillin, and reveal a very low enzymatic activity after osmotic shock. These characters may be used to differentiate periplasmic and cell-bound β-lactamases.

The β-lactam antibiotics (penicillins and cephalosporins) are widely used in the treatment of different infections because of their broad spectrum of antibacterial activity and their lack of toxicity. The utilization of these antibiotics has been reduced due to the increasing frequency of hospital-acquired, gram-negative infections caused by multiresistant strains (4, 21). Production of β-lactamase in gram-negative bacteria is the principal mechanism of resistance to penicillins and cephalosporins (26).

Synthesis of penicillinase can be R factor mediated (8) or can be under the control of a chromosomal gene (12). R factor-mediated penicillinase is a periplasmic enzyme (15) and can be partially released by osmotic shock (18). Penicillinase with chromosomally determined synthesis is firmly bound to the cell (15, 16).

Synergism between β-lactam antibiotics depends upon the competitive inhibition of the β-lactamase by a penicillinase-resistant analogue which thus permits a hydrolyzable penicillin to exert a more prolonged antibacterial effect (11, 24). Combinations of ampicillin and cloxacillin (1, 7) and other selected pairs of β-lactam antibiotics (5, 9, 10, 25) have been proven to be active in vitro and effective for the treatment of urinary tract infections caused by ampicillin-resistant strains. However, all penicillinase-producing strains are not susceptible to the synergistic action of β-lactam antibiotics, and the location of the genes for β-lactamase synthesis, either chromosomal or extrachromosomal, appears to control whether synergism occurs (16).

Enzyme neutralization tests with rabbit antisera, together with substrate profile determinations, have led to the classification of β-lactamases of gram-negative bacteria into different classes and types of enzymes (22, 23).

This paper attempts to correlate different properties of ampicillin-resistant strains of wild-type *Escherichia coli*: the transferability of ampicillin resistance, the release of the enzyme by osmotic shock and the determination of its specific activity, the synergism between ampicillin and cloxacillin, and the substrate profiles of the enzymes and some of their immunological characteristics.

MATERIALS AND METHODS

Bacterial strains. Sixty-four strains of *E. coli*, isolated from urine of patients with urinary tract infections ($\geq 10^5$ bacteria/ml), were studied. The patients were selected from the Geneva hospital. Bacterial strains were classified as resistant to ampicillin by disk test (10 μg/disk). According to their reaction to ampicillin-cloxacillin synergy, 27 strains for which synergy was positive and 37 for which it was negative were selected for the present study.

The resistance pattern for other β-lactam antibiotics was determined by disk tests (carbenicillin [100 μg/disk] and cephalothin [30 μg/disk], using Müller-Hinton agar [Difco] [20 ml/plate], the inoculum being a 1/1,000 dilution of 6-h incubation culture). In our experimental conditions, at 37 C with agitation, bacterial concentration reached about 10^9 to 2×10^9 cells/ml.

MIC. Minimal inhibitory concentrations (MIC) were determined in twofold dilutions of antibiotics (ampicillin, carbenicillin, or cephalothin) prepared in antibiotic medium no. 3 (Penassay broth, Difco). The tubes were inoculated with a 6-h incubation culture giving a final concentration of 1/100 and were incubated at 37 C. The lowest concentration preventing growth (MIC) was estimated after 18 h of incubation. As a control, fully sensitive *E. coli* K-12 strains (K-12 – F^+ – 501 and K-12 – F^- – 703) were tested under the same conditions.

Ampicillin-cloxacillin synergy. This reaction was tested by the cross-strip method of Bonifas (6). One strip was soaked in a solution of ampicillin (3,750 μg/ml) and the other was soaked in a solution of cloxacillin (375 μg/ml). After freeze-drying, the strips were disposed at right angles on a petri dish (9 cm in diameter) containing 20 ml of Müller-Hinton agar (Difco), after being spread with a 1/1,000 dilution of a 6-h incubation culture of the bacterial strain (Fig. 1).

Transfer tests. Transfer tests were done by the method of Anderson and Lewis (3) by using a biparental cross with *Salmonella typhi*, *S. paratyphi* B, and *S. typhimurium* as recipient strains. The selective medium was a salmonella-shigella agar (BBL) containing 100 μg of ampicillin per ml.

Enzyme assay. For all strains, preparation of crude enzyme was performed by osmotic shock by the method of Nossal and Heppel (18). The protein content of the shockates was determined by the method of Lowry et al. (13).

The micro-iodometric determination of Sykes and Nordström (27) was used with all substrates: benzylpenicillin (Specia Laboratories, France), ampicillin, pheneticillin, carbenicillin (Beecham Research Laboratories, United Kingdom), and cephalothin (Eli Lilly & Co., Indianapolis, Ind.). All measurements were done on a DB-GT Beckman spectrophotometer with automatic recording.

Substrate profiles are defined as the relative rate of hydrolysis of the different substrates under standard conditions. Profiles are given as values relative to the rate of hydrolysis of benzyl-pencillin (= 100).

For enzymatic activity, one unit of β-lactamase is defined as the amount which hydrolyzes 1 μmol of substrate (benzyl-penicillin) per min at 30 C and pH 5.8 (19). Specific activity is defined as activity per microgram of total proteins.

Immunological procedures. All procedures for immunological characterization of the β-lactamases were fully described previously (20). The antisera used in this study were prepared from two strains of wild-type ampicillin-resistant *E. coli*, labeled P111 and P453, and correspond to immunotypes 1 and 2, respectively. By using *S. typhi* as recipient strain, transfer frequencies of the ampicillin resistance are 10^{-5} for P111 and 10^{-2} for P453.

RESULTS

Disk diffusion tests. Disk diffusion tests produce three basic types of resistance patterns (Table 1). Group I is formed by 35 strains that are ampicillin resistant (Am^R), cephalothin sensitive (Cf^S), and carbenicillin resistant (Cb^R). Group II includes two strains that are resistant to all three antibiotics (Am^R, Cf^R, Cb^R). Group III contains 27 strains that are ampicillin resistant, cephalothin resistant, and carbenicillin sensitive (Am^R, Cf^R, Cb^S).

MIC. MIC determine the same three groups among the strains studied (Table 1). Ampicillin resistance levels have been found to be much higher for groups I and II (MIC ≥ 1,280 μg/ml) than for group III (MIC 160 to 320 μg/ml). The two strains of group II are resistant to high levels of all three antibiotics. The relatively high MIC values for cephalothin and carbenicillin in strains considered as sensitive in diffusion tests are due to the importance of the inoculum in the MIC determinations. This is confirmed by the MIC values found with the control strains of *E. coli* K-12, the MIC being 10 μg/ml for ampicillin, 20 μg/ml for carbenicillin, and 20 to 40 μg/ml for cephalothin.

Transferability tests. Table 2 shows that, in group I (Am^R, Cf^S, Cb^R), 24 strains out of 35 transfer their ampicillin resistance to the recipient *Salmonella* strains used in these experiments. Transfer was also positive with two strains of group II (Am^R, Cf^R, Cb^R). For the 38 other strains (11 of group I and 27 of group III), it was not possible to detect the presence of a transferable R factor in the classical biparental cross.

Enzymatic specific activity. Thirty-three

TABLE 1. *Resistance pattern and minimal inhibitory concentrations for ampicillin, cephalothin, and carbenicillin in E. coli strains*

Group	No. of strains	Resistance pattern[a]	MIC (μg/ml)[b]		
			Ampicillin	Cephalothin	Carbenicillin
I	35	$Am^RCf^SCb^R$	≥1,280	≤40	≥3,200
II	2	$Am^RCf^RCb^R$	>1,280	1,280	>3,200
III	27	$Am^RCf^RCb^S$	160–320	1,280	100–200

[a] Determined by disk diffusion tests. Abbreviations: Am, ampicillin; Cf, cephalothin; Cb, carbenicillin; R, resistant; S, sensitive.

[b] The lowest concentration preventing growth (MIC) was estimated after 18 h of incubation.

TABLE 2. *Characteristics of ampicillin-resistant E. coli strains*

Group	Subgroup	No. of strains	Transferability of ampicillin resistance	Enzymatic activity (U/μg)[a]	Synergy between ampicillin/cloxacillin	Immunotype				Profile type[c]
						1	2	1 and 2	NT[b]	
I ($Am^RCf^SCb^R$)[d]	a	21	+	79-765	−	16				A
							4			B
								1		B
	b	3	+	59-82	−				3	C
	c	35 8	−	50-278	−	7				A
								1		B
	d	1	−	120	−				1	C
	e	2	−	0.9-1.5	−				2	D
II ($Am^RCf^RCb^R$)[d]		2	+	312-960	−	2				A
III ($Am^RCf^RCb^S$)[d]		27	−	0.07-2.5	+				27	E
										F

[a] U/μg: Units/μg total proteins.
[b] NT, Nontypable by antisera 1 and 2.
[c] The relative rates of hydrolysis for the different substrates are given in Fig. 3 and 4.
[d] Am: Ampicillin, Cf: cephalothin, Cb: carbenicillin, R: resistant, S: sensitive.

strains out of 35 of group I give preparations with a high enzymatic activity by osmotic shock, the specific activity for these strains being between 50 and 765 units/μg of total proteins liberated, by using benzyl-penicillin as substrate. This suggests that, in this group of *E. coli*, β-lactamase has a periplasmic location. This has been shown for other ampicillin-resistant strains of *E. coli* (16). Two strains of this group (subgroup Ie) present a very low enzymatic activity (0.9 and 1.5 units/μg of total proteins). A high enzymatic activity is found with the two strains of group II (Am^R, Cf^R, Cb^R). For the 27 strains of group III (Am^R, Cf^R, Cb^S), the enzymatic activity after osmotic shock is very low, comprising between 0.07 and 2.5 units/μg of total proteins; it may be assumed that, in these strains, the β-lactamase has an intracellular location (15).

Ampicillin-cloxacillin synergy. The 37 strains of groups I and II are indifferent to the synergism between the two antibiotics (Table 2). Figure 1 shows two types of synergism between ampicillin and cloxacillin found with the 27 strains of group III; these two reactions can be correlated to the MIC values in liquid medium.

Immunological characterization. The specific anti-β-lactamase antisera have been prepared from two well-defined plasmidic-resistant strains of *E. coli*, P111 corresponding to immunotype 1 and P453 corresponding to immunotype 2 (20). The 35 strains of group I (Table 2) can be divided into the following

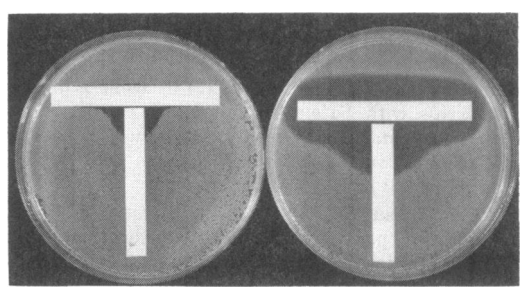

FIG. 1. *Agar diffusion test for synergism between ampicillin (horizontal strip) and cloxacillin (vertical strip) against two E. coli strains. The synergism is demonstrated by the fact that the antibacterial effect of the two drugs together is greater than that of each alone.*

immunotypes: 23 belong to type 1, 4 belong to type 2, and 2 are types 1 and 2. The immunochemical characteristics of enzymes type 1 and 2, as determined by immunoelectrophoresis, are shown in Fig. 2. The remaining six strains are nontypable since the immunoprecipitation tests are negative. The two strains of group II belong to immunotype 1, and the 27 strains of group III are nontypable with both antisera.

Substrate profiles. Figure 3 shows the substrate profiles obtained with strains belonging to groups I and II. Profile A has been determined with seven strains and corresponds to the immunotype 1. Profile B is given by the four strains belonging to immunotype 2. The four strains of group I (Ib and Id) that are nontypable by immunoprecipitation gave profile C. It

can be seen that these three profiles are very similar: only pheneticillin could show a significant difference with hydrolysis rates of 25 for type A, 50 for type B, and 33 for type C. The two strains positive with both antisera present a substrate profile similar to type B. Profile D was determined by the two strains of subgroup Ie. It can be seen that profiles A, B, C, and D correspond to a predominant "penicillinase" activity.

From group III, 8 strains out of 27 have been studied. For profile E (Fig. 4), determined with four strains, no hydrolysis can be detected for ampicillin, pheneticillin, and carbenicillin, whereas cephalothin is slightly destroyed. Profile F (four strains) is characterized by a variable hydrolysis of all the substrates.

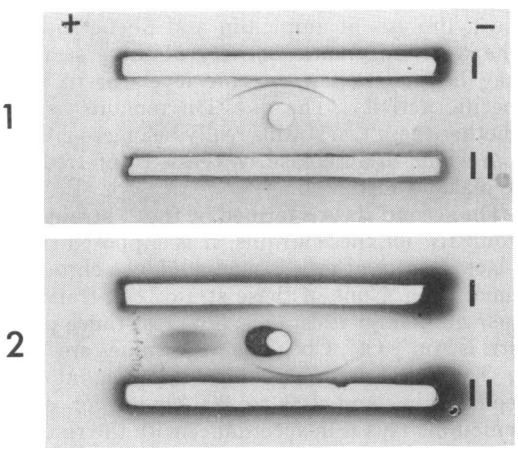

FIG. 2. *Immunoelectrophoretic analysis. 1, Penicillinase from E. coli P111; 2, penicillinase from E. coli P453. I, Antiserum (P111); II, antiserum (P453).*

DISCUSSION

The results obtained in this work may divide the 64 strains studied into two major classes, the first class containing 37 strains of group I and II (Table 2) for which we may assume that β-lactamase synthesis is plasmid mediated. The following arguments support this hypothesis.

Twenty-six of these strains transfer their ampicillin resistance in a biparental cross, using the standard recipient strains of *Salmonella* (3). These strains present a high resistance level to ampicillin (MIC ≥ 1,280 μg/ml) and carbenicillin (MIC ≥ 3,200 μg/ml) but are sensitive to cephalothin (MIC ≤ 40 μg/ml), except for the two strains of group II that are resistant to the three antibiotics. All these strains are indifferent to synergy between ampicillin and cloxacillin. All but three (subgroup Ib) are typable with the two antisera prepared with purified enzyme preparations obtained from two well-defined plasmid-mediated ampicillin-resistant strains (20). With the 26 strains in which resistance to ampicillin is transferable, osmotic shock gives preparations with a high enzymatic activity. These findings are in favor of the hypothesis that, in strains carrying an R factor for resistance to penicillins, the enzyme has a periplasmic location (15, 16). The substrate profiles can be correlated with the immunological characters, since immunotype 1 gives profile A and immunotype 2 gives profile B. The three nontypable enzymes give profile C. The strains of subgroup Ib probably synthesize a plasmid-

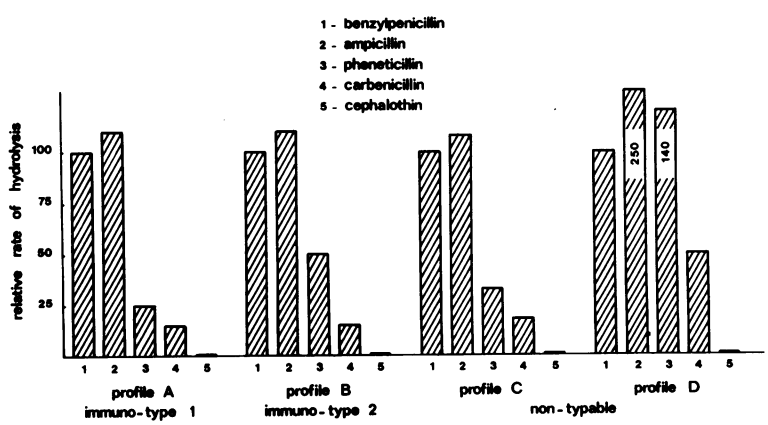

FIG. 3. *Substrate profiles of β-lactamases produced by strains of groups I and II. Ordinate represents relative rate of hydrolysis for each substrate, taking the absolute rate of benzyl-penicillin hydrolysis as 100. Substrate profile type A has been determined with seven strains, substrate profiles type B and type C have been demonstrated with four strains of each type, and substrate profile type D has been demonstrated with two strains.*

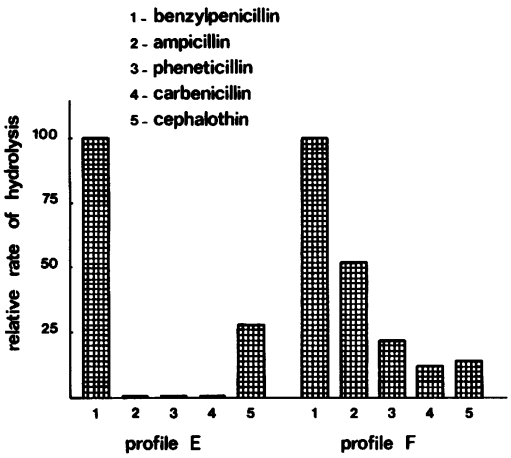

FIG. 4. *Substrate profiles of β-lactamases produced by strains of group III. Ordinate represents relative rate of hydrolysis of each substrate, taking the absolute rate of benzyl-penicillin hydrolysis as 100. Substrate profiles E and F represent the average obtained with four strains each.*

mediated β-lactamase that is immunologically different from enzymes type 1 or 2.

The two strains of group II, resistant to the three drugs, belong to immunotype 1 and give a substrate profile of type A. In the present state of our experiments, the failure to detect enzymatic inactivation of cephalothin in the shockates of high level cephalothin-resistant strains cannot be explained.

All the ampicillin-resistant strains carrying a transferable drug resistance determinant synthesize β-lactamases that correspond to a penicillinase type (Fig. 3, profiles A, B, C).

Extrachromosomal resistance determinants can exist in bacterial cells without the presence of a transfer factor. This has been demonstrated by genetic studies (2, 3) as well as by physical determinations (14). We may then argue that 11 strains for which no transfer of ampicillin resistance is demonstrated may be classified in group I (plasmid-mediated β-lactamases).

Except for transferability of ampicillin resistance, the eight strains of subgroup Ic (Table 2) possess all characteristics of subgroup Ia. They are indifferent to synergy between ampicillin and cloxacillin, and the specific enzymatic activity in their shockates is high. The immunoprecipitation tests show that seven of these strains belong to immunotype 1 and one belongs to immunotypes 1 and 2; their substrate profiles are type A and B, respectively (Fig. 2). These characteristics may suggest that in these strains the R determinants responsible for β-lactamase synthesis are similar to those found in subgroup Ia. According to the model of Anderson (2, 3), we may then suppose that these strains are devoid of a transfer factor.

In the same way, subgroup Id may be affiliated to subgroup Ib; except for transferability of ampicillin resistance, it presents the same characteristics, especially the same profile type C.

The two strains of subgroup Ie present intermediate characteristics; their resistance pattern (Am^R, Cf^S, Cb^R) and their indifference to synergy between ampicillin and cloxacillin classify them in group I. Two characteristics are different: the substrate profile and the enzymatic activity after osmotic shock. The substrate profile type D is primarily a "penicillinase type"; in this respect, it is similar to profiles A, B, and C; however, it differs quantitatively in the hydrolysis of ampicillin and pheneticillin. The weak enzymatic activity of these strains may be due to low enzyme levels or to low specific activity. The question remains as to whether these two strains really belong to group I, in which β-lactamase synthesis is inferred to be plasmid mediated.

The second class is formed by the 27 strains of group III; for these strains, it is supposed that β-lactamase synthesis is mediated by a chromosomal gene. None of these strains can transfer their ampicillin resistance. The resistance pattern is Am^R, Cf^R, Cb^S. The MIC values are 160 to 320 μg/ml for ampicillin, 1,280 μg/ml for cephalothin, and 100 to 200 μg/ml for carbenicillin. This is in accordance with the results presented by Neu (17), who found that *E. coli* and *Salmonella* strains with a low level of ampicillin resistance are sensitive to carbenicillin. All strains of group III are sensitive to synergy between ampicillin and cloxacillin. In spite of the fact that these 27 strains are negative in the immunoprecipitation tests, it is possible to differentiate at least two substrate profiles, E and F (Fig. 4).

The shockates obtained from the second class of *E. coli* strains present a very weak enzymatic activity, since the range is comprised between 0.07 and 2.5 units/μg of total proteins. This implies either that the enzyme is an internal β-lactamase, not released by osmotic shock (15, 16), or that the specific activity or the enzyme level is low.

The results obtained in this work confirm that synthesis of β-lactamases in wild-type strains of *E. coli* may be mediated either by a chromosomal gene or by an extrachromosomal element. The substrate profiles of R factor penicillinase-producing strains corroborate their resistance pattern. This fact suggests that β-lactamase production is the main factor of

penicillin resistance. For strains in which β-lactamase synthesis is directed by a chromosomal gene, discrepancies can be seen between substrate profiles and resistance pattern. Possible interactions between β-lactamases and "intrinsic" resistance mechanisms may be involved (23).

These observations imply that synergy between ampicillin and cloxacillin, combined with the resistance pattern for ampicillin, cephalothin, and carbenicillin may be used to differentiate periplasmic and cell-bound β-lactamases. The majority of ampicillin-resistant strains of *E. coli* are sensitive to cephalothin or carbenicillin. Strains which are susceptible to the synergistic action of ampicillin and cloxacillin are sensitive to carbenicillin and to low level of ampicillin ($\leq$ 320 μg/ml); this level can be achieved in the urine with therapeutic doses of antibiotic.

For the treatment of urinary tract infections due to *E. coli* strains producing chromosomally mediated β-lactamases, it might be justifiable, on bacteriological grounds, to propose the association of synergistic pairs of β-lactam antibiotics. However, such an association would be useless where the strains implicated in the infection synthesize an R factor-mediated β-lactamase. It must be noted that the proportion of plasmid-controlled β-lactamases in ampicillin-resistant strains of *E. coli* represents 80% of the total clinical isolates.

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