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Selection and Characterization of β-Lactam-Resistant *Escherichia coli* K-12 Mutants

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β-Lactam-resistant mutants of *Escherichia coli* K-12 were selected by using 12 different β-lactam derivatives. The mutants fell into three categories showing (i) altered permeation through reduction or loss of outer membrane porin proteins (including *ompF*, *ompR*, and *envZ* alleles); (ii) increase in the rate of synthesis of chromosomally mediated β-lactamase; or (iii) defective synthesis or action of cyclic adenosine 3',5'-phosphate (*cya* and *crp* alleles).

In a previous report (10), we described mutants of *Escherichia coli* K-12 that are resistant to the β-lactam antibiotic cefoxitin. We demonstrated that they are mutated in the regulatory genes *ompR* (previously called *ompB*) and *envZ* (previously called *tpo*) and in the structural gene *ompF*, whose products are required for the expression of two major outer membrane proteins, OmpF and OmpC (18). These proteins form pores in the outer membrane that allow small molecules like the β-lactams to diffuse rapidly into the periplasmic space (13). Diminished levels of only OmpF or of both OmpF and OmpC porin proteins enhance the resistance of *E. coli* K-12 towards β-lactams (2, 9, 10, 19). Mutants of this type were obtained spontaneously at high frequency and may be of interest because they are resistant to clinically useful β-lactam antibiotics. The study of the mechanisms conferring resistance to β-lactam antibiotics is important in the prediction of resistant mutants that could emerge after therapy. Many new cephalosporins with broad antibacterial spectra have been developed in the past few years. In this work we study the physiological implications of spontaneous mutants of *E. coli* K-12 that could be isolated in laboratory culture by using 12 β-lactam antibiotics as selectors. We have characterized these β-lactam-resistant mutants genetically and physiologically.

Three independent cultures of *E. coli* K-12 strain pop1010 (20) were grown overnight in rich medium, and samples were plated on plates containing specific amounts of antibiotic (Table 1) and incubated at 37°C. For each selector, a total of nine clones (three clones per culture) were purified and analyzed. The tests used were (i) susceptibility to phages TuIa, 434, TP1, and K-10, which distinguish *ompR*, *ompF*, and *envZ* mutants (6, 8, 20, 21); (ii) antibiotic susceptibility as measured by 99% inhibitory concentrations (5); (iii) β-lactamase activity (17); (iv) the presence or absence of OmpF and OmpC porin proteins, as determined from gels of total cell proteins (14); (v) the penicillin-binding protein pattern, as determined by the method of Spratt and Pardee (15); and (vi) genetic localization of the mutations by P1 transduction analysis. Genetic and biochemical techniques were described previously (10).

The frequencies and the classifications of the mutants selected in the presence of specific antibiotic concentrations are reported in Table 1. The mutants isolated on ampicillin (5 μg/ml), carbenicillin, cephaloridine, cefazolin, cefotaxime, cefoxitin, furazlocillin (3, 22), azthreonam (16), moxalactam, and SCH29482 (4), were all affected in the production of the OmpF and OmpC porin proteins. Genetically they fell into three subclasses, as established by P1 transduction: *ompR* (74 min on the genetic map), *ompF* (21 min), and *envZ* (74 min). Mutants selected on carbenicillin, cefotaxime, and furazlocillin showed a wild-type phenotype with unaltered phage susceptibility and unaltered gel electrophoresis pattern. We failed to detect cotransduction between the resistance character and markers known to be linked to the *ompR*, *envZ*, and *ompF* mutations, and the precise location of these mutations has not been determined. The failure to detect cotransduction might be due to the existence of another gene(s) controlling antibiotic permeation.

Mutants selected in the presence of a higher concentration of ampicillin (20 μg/ml) were found at low frequency. They were affected in the production of chromosomally determined β-lactamase. Total β-lactamase activity was 50 times higher in these mutants than in the wild type. The genetics of ampicillin resistance in *E. coli* has been extensively studied. Hyperproduction of β-lactamase can be acquired either by

TABLE 1. Frequency and distribution of resistant mutants

Selective antibiotic	Concn (μg/ml)	Frequency of resistant mutants	No. of mutants							
			ompR	*ompF*	*envZ*	ND[a]	*cya*	*crp*	*amp*[b]	Unstable
Ampicillin	5	8×10^{-7}		6	2					1
	20	5×10^{-9}							4	5
Carbenicillin	10	2×10^{-7}				9				
Cefaloridin	10	10^{-8}	9							
Cefazolin	10	10^{-8}	9							
Cefotaxime	0.08	2×10^{-7}				9				
Cefoxitin	10	9×10^{-7}	8	1						
Furazlocillin	0.25	2×10^{-7}				9				
Azthreonam	0.064	3×10^{-6}		5	2					2
Moxalactam	0.5	10^{-8}	9							
SCH29482	0.4	10^{-6}	7		1					1
N-Formimidoyl thienamycin	0.5	10^{-6}					5			4
Mecillinam	5	6×10^{-5}					7	1		1

[a] ND, Not determined.
[b] Enhanced β-lactamase production.

repetition of the structural gene *ampC* (7) or by mutation in the control sequence region of *ampC* (11). The mutants studied here were shown by P1 transduction to carry mutations in the *ampC* region (94 min).

Mutants selected on the antibiotics mecillinam or *N*-formimidoyl thienamycin were mutated in the genes coding for the cAMP receptor protein (*crp*, 73 min) or adenylate cyclase (*cya*, 84 min). This was established by their Lac^- Mal^- phenotype, by their resistance to phage λ, and by P1 transduction. It was reported earlier that *crp* and *cya* mutants were highly resistant to mecillinam (1). We confirmed this result and extended it to the very active antibiotic *N*-formimidoyl thienamycin. Mecillinam is known to bind specifically to penicillin-binding protein 2 in the inner membrane of *E. coli* (15), but the regulation of penicillin-binding protein 2 synthesis is unknown. We could not detect any differences in the pattern of penicillin-binding proteins in the *crp* and *cya* mutants compared with the parental strain.

Unstable clones were also found with several selectors. These clones appeared when overnight cultures of susceptible bacteria were plated on selective media but did not grow further during purification on plates containing the same amount of the same antibiotic. Table 2 shows the antibiotic susceptibility of the strains, measured by the inhibitory concentration that reduced CFU by 99%. The 99% inhibitory concentrations for the parental strain and for one or two representative strains of each type of mutant are shown in Table 2. The *ompR* mutation, which reduces the production of both porin proteins OmpF and OmpC, decreased the susceptibility of *E. coli* K-12 by a factor of 2 to 16, depending on the antibiotic, except for *N*-formimidoyl thienamycin, whose 99% inhibitory concentration remained equal to that of the parental strain. The decreased production of the OmpF porin protein in *ompF* and *envZ* mutants reduced the susceptibility towards the series of antibiotics less than it did in the *ompR* mutants.

The *ompC* mutant B1478, constructed by transduction, has been incorporated in Table 2 to show, as discussed previously (10), that the absence of the OmpC porin alone does not alter the antibiotic susceptibility of *E. coli* K-12. The increased β-lactamase production in strain B1531 served primarily to reduce susceptibility to ampicillin. Strains B1540 (*cya*) and B1558 (*crp*) showed essentially no change in susceptibility to any of the β-lactam antibiotics tested except furazlocillin, *N*-formimidoyl thienamycin (for B1540), and (especially) mecillinam, whose 99% inhibitory concentration was increased by a factor of 100.

In conclusion, it appears that the β-lactam

TABLE 2. Susceptibility to β-lactams

Strain	Selector	Mutation	99% Inhibitory concentration (μg/ml) of:											
			Ampicillin	Carbenicillin	Cephaloridine	Cefazolin	Cefotaxime	Cefoxitin	Furazlocillin	Azthreonam	Moxalactam	SCH29482	*N*-Formimidoyl thienamycin	Mecillinam
B1389		Wild type	2	2	2	1–2	0.03	2	0.06	0.06	0.06	0.5	0.125	0.125
B1478		*ompC*	2	2	2	1	0.03	2	0.06	0.06	0.06	0.5	0.125	0.125
B1554	Cephaloridine	*ompR*	4	4	16	8	0.06	16	0.125	0.125	1	2	0.125	0.125
B1567	Azthreonam	*ompF*	4	4	4	2	0.06	8	0.06	0.125	0.5	1	0.125	0.25
B1563	Cefotaxime	ND[a]	8	8	4	2	0.125	16	0.25	0.125	0.25	0.5	0.125	0.25
B1541	Carbenicillin	ND	2	32	2	2	0.03	4	0.06	0.5	1	0.25	0.125	0.125
B1569	SCH29482	*envZ*	8	8	2	2	0.06	8	0.25	0.25	1	1	0.5	1
B1528	Ampicillin (5 μg/ml)	*envZ*	8	4	2	1	0.06	4	0.125	0.06	0.125	0.5	0.125	0.25
B1540	*N*-Formimidoyl thienamycin	*cya*	4	4	4	2	0.03	2	0.5	0.06	0.125	1	1	128
B1558	Mecillinam	*crp*	4	4	4	1	0.03	2	0.125	0.125	0.125	0.5	0.5	128
B1531	Ampicillin (20 μg/ml)	*amp*	32	4	8	2	0.125	8	0.125	0.25	0.06	0.25	0.125	0.125

[a] ND, Not determined.

derivatives ampicillin, cephaloridine, cefazolin, cefoxitin, azthreonam, moxalactam, and SCH29482 provide a ready selection for mutants altered in the mechanism of permeation. Porin-deficient resistant mutants of *E. coli* were previously isolated by other workers (2, 10, 19) but were not characterized genetically. We have shown here that decreased penetration of these β-lactams through the outer membrane of *E. coli* K-12 can be achieved through at least three different mutations on the chromosome. Cephaloridine, cefazolin, and moxalactam seemed specifically to select *ompR* mutations. This could reflect a higher inherent permeability of these compounds, as proposed by Nikaido (12). *N*-Formimidoyl thienamycin and mecillinam, on the other hand, did not select permeation mutants but rather mutants defective in the synthesis or action of cAMP, which might regulate the activity of penicillin-binding protein 2 in some unknown way.

From the clinical point of view, the critical values which define the resistance of a strain to β-lactams are usually more than 32 μg/ml. According to this definition, only *cya* and *crp* mutants would be classified as mecillinam-resistant strains. Mutants resistant to 8 μg of various β-lactams per ml belong to the intermediately susceptible class and might reduce the therapeutic activity of these derivatives. In addition, porin mutations occuring in strains harboring plasmid-mediated β-lactamase might greatly enhance their resistance. It would be interesting to see whether permeability mutants, which in laboratory cultures represent the major class of β-lactam-resistant *E. coli* mutants, could be detected among intermediately susceptible enterobacterial strains in nature.

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