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Correlation of Resistance to Proflavine and Penicillin in *Escherichia coli*

ROBIN C. McKELLAR, COLIN N. McKENZIE, AND DONN J. KUSHNER*
Department of Biology, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5

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A number of proflavine (PF)-resistant mutants of *Escherichia coli* B were also resistant to penicillin and cephalothin. Mutants resistant to 1.0 mM PF were 10 times more penicillin resistant than were the PF-susceptible, wild-type cells. Single-step mutants selected for resistance to either PF or penicillin were also resistant to the other drug. None of the resistant mutants tested possessed β-lactamase activity. These results suggest that resistance to PF and penicillin in *E. coli* B may be due to permeability changes in the cell envelope.

The role of permeability in bacterial resistance to acridine dyes is uncertain. Earlier reports associated resistance of different strains of *Escherichia coli* to proflavine (PF) and acriflavine (AF) with decreased dye uptake (1, 5, 6, 14, 20, 21, 25). The fact, however, that resistant cells can actively expel acridines has complicated interpretations of experiments based on uptake alone (7, 14).

Work in our laboratory previously suggested that resistance occurred at the metabolic level, since glucose metabolism was inhibited by PF in susceptible but not in resistant *E. coli* (10). It was since found that pyruvate kinase I may be the site of inhibition of glycolysis by PF in sensitive cells. However, purified pyruvate kinase I from susceptible and resistant cells was equally susceptible to PF (unpublished data). These results suggest again that permeability is involved in PF resistance, at least in the sense that some PF-sensitive site(s) may be shielded from the drug by cell structures.

Permeability changes have been implicated as the basis of cross-resistance between unrelated inhibitors. AF resistance due to permeability barriers has been associated with resistance to methanol and thiabendazole in *Dictyostelium discoideum* (24) as well as with resistance to phenethyl alcohol and sodium dodecyl sulfate in *E. coli* (12).

Acridine orange resistance was associated with resistance to penicillin, erythromycin, chloramphenicol, rifampin, and ethidium bromide in *Neisseria gonorrhoeae* (8).

We report here a study of the relation between resistance to PF and penicillin in a number of strains of *E. coli*.

Strains resistant to 1.0 mM PF were isolated from *E. coli* B after growth in Trypticase soy broth (TSB; Baltimore Biological Laboratory [BBL], Cockeysville, Md.) containing successively 0.02, 0.10, 0.20, 0.50, and 1.0 mM PF as previously described (7). Strains resistant to 0.1 mM PF were isolated from Trypticase soy agar (TSA [TSB plus 1.5% agar]; Difco Laboratories, Detroit, Mich.) containing 0.1 mM PF that had been heavily inoculated with *E. coli* B. For these and other experiments, inocula consisted of overnight cultures grown in TSB at 37°C.

Several 1.0 mM PF-resistant strains were exposed to a variety of antibiotics (obtained from BBL and Difco) on TSA plates containing 4 to 5 antibiotic disks/plate. After incubation, susceptibility or resistance was measured as the width of the clear zone surrounding the disk.

All PF-resistant strains exhibited increased resistance to penicillin, cephalothin, and, to a lesser extent, ampicillin (Table 1). PF-susceptible and -resistant strains were equally susceptible to most of the other antibiotics.

To determine the level of PF-associated penicillin resistance, individual strains were inoculated into tubes containing 10 ml of TSB and different concentrations of penicillin. After incubation, growth was measured as absorbance at 660 nm in a Coleman Jr. spectrophotometer. After 24 h, strains resistant to 1.0 mM PF displayed a 10-fold increase in the minimal inhibitory concentration (that is, the lowest concentration completely preventing growth) of penicillin (from 15 to 150 U/ml), whereas those resistant to 0.1 mM PF possessed intermediate levels of penicillin resistance (Fig. 1). These measurements do not distinguish between killing and growth inhibition by penicillin. After 44 h, further growth had taken place in some tubes, but the same pattern still held (minimal inhibitory concentrations, 25 U of penicillin per

TABLE 1. *Susceptibility of PF-resistant strains of E. coli B to antibiotics*[a]

Antibiotic (amt/disk)	Width of clear zone (mm)				
	B[b]	B/Pr	PrA	PrB	PrC
Penicillin, 10 U	2.33 ± 0.11 (6)	0 (7)	0 (4)	0 (4)	0 (4)
Cephalothin, 30 μg	6.58 ± 0.08 (3)	1.00 ± 0.14 (3)	0 (5)	1.20 ± 0.09 (5)	1.50 ± 0.35 (4)
Ampicillin 10 μg	8.33 ± 0.17 (3)	3.63 ± 0.13 (2)	3.0 ± 0.20 (4)	4.90 ± 0.19 (5)	4.75 ± 0.14 (4)
Carbenicillin, 100 μg	10.83 ± 0.17 (3)	4.25 ± 0.10 (4)	7.25 ± 0.14 (4)	6.70 ± 0.12 (5)	6.91 ± 0.15 (6)
Chloramphenicol, 5 μg	2.95 ± 0.10 (6)	0 (6)	2.25 ± 0.14 (4)	1.50 ± 0 (4)	2.63 ± 0.24 (4)
Tetracycline, 30 μg	3.19 ± 0.18 (9)	2.63 ± 0.25 (9)	2.25 ± 0.14 (4)	1.10 ± 0.16 (4)	1.00 ± 0 (4)
Streptomycin, 10 μg	1.60 ± 0.10 (5)	1.75 ± 0.30 (4)	2.00 ± 0.35 (4)	0.67 ± 0.17 (3)	2.63 ± 0.13 (5)
Rifampin, 10 μg	2.50 ± 0 (2)	0.88 ± 0.13 (2)	1.60 ± 0.10 (5)	1.62 ± 0.13 (4)	1.38 ± 0.24 (4)
Gentamicin, 10 μg	1.58 ± 0.08 (3)	1.80 ± 0.17 (3)	NT	NT	NT
Nitrofurodantin, 30 μg	5.80 ± 0.08 (3)	6.00 ± 0.22 (3)	NT	NT	NT
Neomycin, 10 μg	1.50 ± 0 (5)	2.20 ± 0.17 (3)	NT	NT	NT
Septra, 10 μg	4.83 ± 0.17 (3)	3.17 ± 0.73 (3)	NT	NT	NT
Nalidixic acid, 30 μg	4.25 ± 0.14 (3)	4.83 ± 0.17 (3)	NT	NT	NT
Colistin, 10 μg	2.50 ± 0 (3)	3.30 ± 0.08 (3)	NT	NT	NT

[a] Strain B is PF susceptible, and the others are resistant to 1.0 mM PF. Values are presented with standard errors. Bracketed figures represent the number of determinations. NT, Not tested.
[b] Strain.

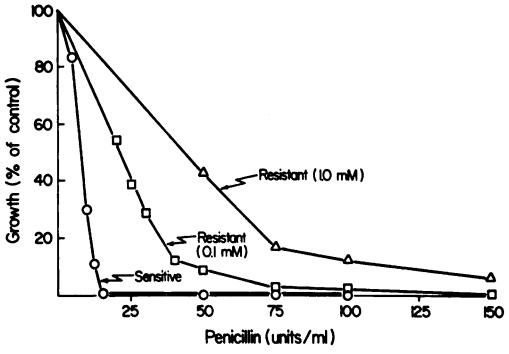

FIG. 1. *Effect of penicillin concentration on growth of PF-susceptible and -resistant E. coli B. Cells were grown in 10 ml of TSB at 37°C for 24 h. Growth was determined as absorbance at 660 nm. One hundred percent growth corresponded to an absorbance of 0.80 at 660 nm.*

ml for the PF-susceptible strain and 300 U/ml for the strain resistant to 1.0 mM PF).

Correlation between PF and penicillin resistance was also studied by replica plating. When 55 colonies of *E. coli* B on TSA not previously exposed to either drug were transferred to a TSA plate containing 25 U of penicillin per ml, no colonies appeared. A similar transfer to TSA containing 0.1 mM PF produced two colonies. Thirteen penicillin-resistant colonies were obtained by spreading 0.5 ml of an overnight culture of *E. coli* B on plates containing 25 U of penicillin per ml and incubating a further 24 h. Twelve of the 13 penicillin-resistant colonies were PF resistant. In a separate experiment 64 PF (0.1 mM)-resistant colonies were obtained in a similar fashion; 54 of these were also resistant to penicillin.

Penicillin resistance is often due to the production of β-lactamase enzymes by resistant bacteria (3, 9, 18); however, in some cases, resistance may result from changes in cell envelope permeability associated with modifications of the lipopolysaccharide layer of the cell wall (2, 11, 15, 16, 19, 22, 23). Such "intrinsic" resistance (29) may occur in cells that lack β-lactamase activity (3).

Three strains resistant to 1.0 mM PF were tested for β-lactamase activity (17). Cells were grown to early log phase in 0.5% glucose mineral salts media (7) at 37°C. In some experiments, penicillin was added to a final concentration of 25 U/ml, and growth was allowed to continue for a further 3 h. PF-susceptible cells lysed within 30 min of penicillin addition, whereas the growth of PF-resistant cells was unaffected by the antibiotic. None of the PF-resistant strains possessed β-lactamase activity, as measured in whole cultures, either untreated or broken by ultrasonic treatment, after growth in the presence or absence of penicillin.

It seems likely from these results that penicillin resistance in *E. coli* B is intrinsic. Since penicillin resistance seems well correlated with PF resistance, we suggest that both phenomena may be caused by permeability changes in the cell envelope.

The study of PF resistance in *E. coli* B/Pr has been complicated by the diversity of binding sites available to PF (4) as well as by the energy-dependent release of bound dye (7, 14). Gross permeability differences to PF do not exist between PF-susceptible and -resistant strains of *E. coli* B (4, 7). It is possible, however, that subtle permeability changes have taken place that are not detectable in PF-binding studies. Nakamura has shown that AF-susceptible and -resistant *E. coli* K-12 bind sim-

ilar amounts of PF (13), in spite of the fact that AF-resistant cell membranes lack a specific structural protein involved in AF uptake and binding (14).

It now seems possible that *E. coli* mutants selected for resistance to either PF or penicillin have undergone changes in an envelope component(s) that make them resistant to both drugs.

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LITERATURE CITED

1. **Barabas, G., B. M. Mehta, and D. J. Kushner.** 1970. Proflavine binding and release by sensitive and resistant *Bacillus subtilis*. Can. J. Microbiol. 16:973–981.
2. **Barrett, E., and A. W. Asscher.** 1972. Action of ethylenediamine tetraacetic acid (EDTA) on carbenicillin-resistant strains of *Pseudomonas aeruginosa*. J. Med. Microbiol. 5:355–359.
3. **Citri, N.** 1971. Penicillinase and other β-lactamases, p. 23–46. *In* P. D. Boyer (ed.), The enzymes, vol. 4. Academic Press Inc., New York.
4. **Gravelle, M. J., B. M. Mehta, and D. J. Kushner.** 1972. The elusive permeability barriers and binding sites for proflavine in *Escherichia coli*. Antimicrob. Agents Chemother. 1:470–475.
5. **Hessler, A. Y.** 1965. Acridine resistance in bacteriophage T2H as a function of dye penetration measured by mutagenesis and photoinactivation. Genetics 52:711–722.
6. **Kohno, T., and J. R. Roth.** 1974. Proflavine mutagenesis of bacteria. J. Mol. Biol. 89:17–32.
7. **Kushner, D. J., and S. R. Khan.** 1968. Proflavine uptake and release in sensitive and resistant *Escherichia coli*. J. Bacteriol. 96:1103–1114.
8. **Maness, M. J., and P. F. Sparling.** 1973. Multiple antibiotic resistance due to a single mutation in *Neisseria gonorrhoeae*. J. Infect. Dis. 128:321–330.
9. **Massari, S., P. Dell' Antone, R. Colonna, and G. F. Azzone.** Mechanism of atebrin fluorescence changes in energized submitochondrial particles. Biochemistry 13:1038–1043.
10. **Mehta, B. M., M. J. Gravelle, and D. J. Kushner.** 1973. Effects of energy metabolism on the release of bound proflavine from sensitive and resistant *Escherichia coli*. Antimicrob. Agents Chemother. 4:332–336.
11. **Monner, D. A., S. Jonsson, and H. G. Boman.** 1971. Ampicillin-resistant mutants of *Escherichia coli* K-12 with lipopolysaccharide alterations affecting mating ability and susceptibility to sex-specific bacteriophages. J. Bacteriol. 107:420–432.
12. **Nakamura, H.** 1968. Genetic determination of resistance to acriflavine, phenethyl alcohol, and sodium dodecyl sulfate in *Escherichia coli*. J. Bacteriol. 96:987–996.
13. **Nakamura, H.** 1974. Plasmid-instability in Acr A mutants of *Escherichia coli* K-12. J. Gen. Microbiol. 84:85–93.
14. **Nakamura, H., and A. Suganuma.** 1972. Membrane mutation associated with sensitivity to acriflavine in *Escherichia coli*. J. Bacteriol. 110:329–335.
15. **Nelson, B. W., and R. J. Roantree.** 1967. Analysis of lipopolysaccharides extracted from penicillin-resistant, serum-sensitive *Salmonella* mutants. J. Gen. Microbiol. 48:179–188.
16. **Nordström, K., L. G. Burman, and K. G. Eriksson-Grennberg.** 1970. Resistance of *Escherichia coli* to penicillins. VIII. Physiology of a class II ampicillin-resistant mutant. J. Bacteriol. 101:659–668.
17. **Novick, R. P.** 1962. Micro-iodometric assay for penicillinase. Biochem. J. 83:236–240.
18. **Richmond, M. H., G. W. Jack, and R. B. Sykes.** 1971. The β-lactamases of gram-negative bacteria including *Pseudomonads*. Ann. N.Y. Acad. Sci. 182:243–257.
19. **Richmond, M. H., and R. B. Sykes.** 1973. The β-lactamases of gram negative bacteria and their possible physiological role. Adv. Microb. Physiol. 9:31–88.
20. **Silver, S.** 1965. Acriflavine resistance: a bacteriophage mutation affecting the uptake of dye by the infected bacterial cells. Proc. Natl. Acad. Sci. U.S.A. 53:24–30.
21. **Silver, S., E. Levine, and P. M. Spielman.** 1968. Acridine binding by *Escherichia coli*: pH dependency and strain differences. J. Bacteriol. 95:333–339.
22. **Suginaka, H., A. Ichikawa, and S. Kotani.** 1974. Penicillin-resistant mechanisms in *Pseudomonas aeruginosa:* effects of penicillin G and carbenicillin on transpeptidase and D-alanine carboxypeptidase activities. Antimicrob. Agents Chemother. 6:672–675.
23. **Weiser, R., A. W. Asscher, and J. Wimpenny.** 1968. In vitro reversal of antibiotic resistance by ethylenediamine tetraacetic acid. Nature (London) 219:1365–1366.
24. **Williams, K. L., R. H. Kessin, and P. C. Newell.** 1974. Parasexual genetics in *Dictyostelium discoideum*: mitotic analysis of acriflavine resistance and growth in axenic medium. J. Gen. Biol. 84:59–69.
25. **Woods, D. R., V. R. Schauder, and P. B. Waddington.** 1973. Acriflavine uptake and resistance in *Serratia marcescens* cells and spheroplasts. J. Bacteriol. 114:59–64.