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Mode of Incomplete Cross-Resistance Among Pipemidic, Piromidic, and Nalidixic Acids

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Spontaneous mutants with various patterns of resistance to pipemidic acid (PPA), piromidic acid (PA), and nalidixic acid (NAL) were isolated from *Escherichia coli* K-12. Most mutants were less resistant to PPA than to PA and NAL, and some mutants resistant to PA and NAL were hypersusceptible to PPA. As for the mutants tested, resistance to the drugs was conferred by mutations at *nalA* and new *nal* genes designated as *nalC* and *nalD*, both of which were located at about 82 min on the recalibrated map. Resistance to PA and NAL was due to decreased sensitivity of the bacterial DNA synthesizing system to them and insufficient drug transport, whereas resistance to PPA was only due to the former.

Pipemidic acid (PPA) (11) is a synthetic antibacterial agent mainly active against gram-negative bacteria, including *Pseudomonas aeruginosa* (15). It is structurally related to piromidic acid (PA) (14) and nalidixic acid (NAL) (10) as shown in Fig. 1. Cross-resistance among the drugs is so incomplete that bacteria highly resistant to PA and NAL are inhibited by PPA at relatively low concentrations (15). To study this incomplete cross-resistance, spontaneous mutants with various patterns of resistance to the drugs were isolated from *Escherichia coli* K-12. Most mutants were less resistant to PPA than to PA and NAL, and some mutants resistant to PA and NAL were, on the contrary, more susceptible to PPA than the parent strain. Genetic analysis of several mutants showed that resistance to these drugs was conferred by *nalA* (7) and new *nal* genes, designated as *nalC* and *D*, mapped at about 82 min on the recalibrated linkage map of *E. coli* K-12, (1). These genes determined sensitivity of the bacterial DNA synthesizing system to the drugs with or without potentiation of ethylenediaminetetraacetate (EDTA) effect.

Incomplete cross-resistance among PPA, PA, and NAL would be accounted for by differences in resistance genes, drug sensitivity of the bacterial DNA synthesizing system, and drug transport.

MATERIALS AND METHODS

Bacteria and phage. All of the bacteria used are derivatives of *E. coli* K-12. The strains KL-16 (Hfr, *thi relA*), JC1552 (F^-, *leu trp his argG metB lacY gal malA xyl mtl strA tonA tsx supE*), and AB3505 (F^-, *proA trp his ilvD metE argH lacY* or *Z galK xyl mtl malA tsx*) were obtained from B. J. Bachmann. The strain 5199, a *thyA* derivative of JC1552, was made by trimethoprim selection. Spontaneous mutants resistant to PPA, PA, and NAL (N-21, N-24, N-31, N-51, P-5, and P-18) were isolated from *E. coli* KL-16 by plating it on nutrient agar containing PPA or NAL at graded concentrations. P1*kc* was supplied by H. Ogawa.

Drugs. PPA, PA, and NAL were produced as described previously (11, 14) in our laboratories. Equimolar NaOH was added for the dissolution of these compounds in water. Streptomycin sulfate was a product of Dainippon Pharmaceutical Co., Ltd. [6-^{3}H]thymidine (25 Ci/mmol) was purchased from The Radiochemical Centre.

Media. Nutrient agar was purchased from Eiken Co., Ltd. F-top agar, R-top agar, and LB medium or agar (12), L broth (9), and a minimal medium (3) were prepared as described previously. Supplements were added, if necessary, at concentrations (micrograms per milliliter) of: L-proline, 20; L-tryptophan, 10; L-arginine-HCl, 40; L-histidine, 20; L-isoleucine, 20; L-valine, 20; L-methionine, 20; thiamine-HCl, 1; thymine, 50; and D-sugars, 5,000.

Measurement of drug susceptibility. The minimal inhibitory concentration (MIC) was determined by the twofold agar-dilution method, where one loopful of 10^3 dilutions of overnight cultures was inoculated onto nutrient agar containing the drugs at graded concentrations and incubated at 37°C overnight. The colony-forming ability of bacteria was tested by plating them with 10 ml of nutrient agar containing the drugs at graded concentrations and incubating at 37°C for 2 days.

Conjugation and transduction. For conjugation, fresh male and female cultures in LB medium (about 2×10^8 cells/ml each) were mixed at a volume ratio of 1 to 2, gently shaken for 5 min, diluted 10 times with the same prewarmed medium, and gently agitated at 37°C throughout the course of mating. The procedure for interrupted matings was as described by Hane and

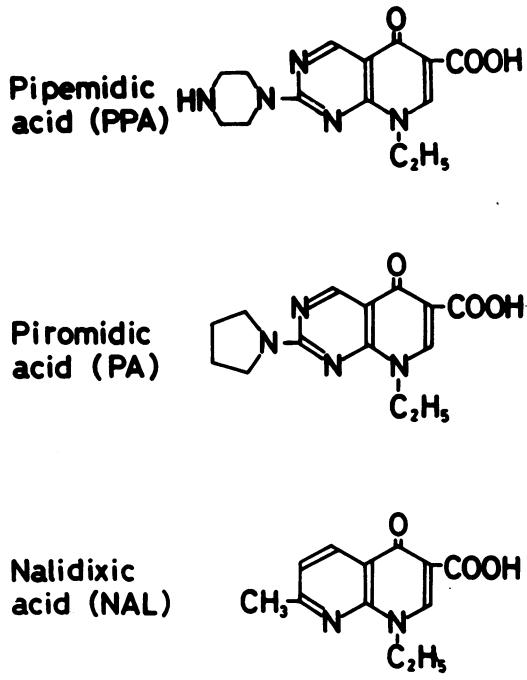

FIG. 1. *Chemical structures of PPA, PA, and NAL.*

Wood (7) except that samples taken periodically were blended before mixing with soft agars, and the media used were F-top agar and the minimal medium supplemented with appropriate nutrients for nutrient selection, and R-top agar and LB agar for drug resistance selection. For transduction, recipient cells freshly cultured in L broth and suspended in 1/10 volume of MC buffer (about 3×10^8 cells/ml) were transduced with P1*kc* by the method of Miller (12). In these matings and transduction, the selective drug concentrations used were NAL, 50 μg/ml for N-51; NAL, 25 μg/ml for N-21 and N-31; NAL, 12.5 μg/ml for N-24; and PPA, 3.13 μg/ml for P-18 and P-5. Check of unselected markers of recombinants and transductants was accomplished by the multiple-loop inoculator method, in which colonies were suspended in 0.05 ml of saline in the wells of microtiter trays and the suspensions were inoculated onto indicator plates with a multiple-loop inoculator carrying about 1 μl per loop.

DNA synthesis. Bacterial cells freshly grown in the minimal medium supplemented with thiamine-hydrochloride and desoxyadenosine (0.2 mM) were treated with EDTA by the procedure of Leive (8). EDTA-treated and untreated cells were diluted 10 times with the above medium to a cell density of about 3×10^8 cells per ml and incubated at 37°C for 4 min. The cell suspensions (0.85 ml) were mixed with 0.05 ml of [^{3}H]thymidine (0.1 μCi) and 0.1 ml of a drug solution and incubated at 37°C for 20 min. The cells precipitated with 5 ml of cold 5% trichloroacetic acid were washed three times with the acid, dissolved in 0.5 ml of Soluene 350 (Packard), neutralized with 1 drop of glacial acetic acid, mixed with 10 ml of toluene scintillator (6), and assayed for radioactivity with a Tri-Carb liquid scintillation spectrometer (Packard, model 3380). Fifty percent inhibitory dose (ID_{50}) of DNA synthesis was calculated graphically.

RESULTS

Mutants isolated. The MICs of PPA, PA, and NAL for the original strain, KL-16, were 1.56, 12.5, and 3.13 μg/ml, respectively. The MIC spectra of mutants isolated on plates containing PPA or NAL ranged from 0.39 to 25 μg/ml for PPA; from 12.5 to over 100 μg/ml for PA, and from 6.25 to over 100 μg/ml for NAL. Most mutants were less resistant to PPA than to PA and NAL irrespective of the selective agents used. Some mutants selected on NAL plates were resistant to PA and NAL but hypersusceptible (more susceptible than KL-16) to PPA.

Several representative mutants were selected and tested on their colony-forming ability in the presence of the drugs at graded concentrations (Fig. 2). The colonies formed of KL-16 were reduced by more than 99% with 0.78 μg of PPA per ml, 12.5 μg of PA per ml, and 3.13 μg of NAL per ml. N-51 and P-18 were 16 and 8 times more resistant to PPA than KL-16, more than 32 times more resistant to PA, and 128 times more resistant to NAL. P-5 and N-24 were 8 or 16 times more resistant to these three drugs. N-21 and N-31 were 2 times more susceptible to PPA than KL-16, whereas they were 8 and 32 times more resistant to PA and NAL, respectively. Thus, the levels of resistance to PPA were generally less than those to PA and NAL.

Mapping of resistance genes. In interrupted matings of the mutants, which transferred chromosomes counterclockwise with an origin at approximately 60.5 min, with a female, 5199, NAL resistance of N-51, and PPA resistance of P-18 and P-5 entered at about 13 min after *thy* and about 4 min before *his*. Therefore, the resistance genes are considered to be at about 48 min on the map of *E. coli* K-12 (1), where *nalA* is situated.

The levels of resistance to PPA, PA, and NAL of the recombinants tested were the same as those of the donors. On the other hand, NAL resistance of N-21, N-31, and N-24 did not transfer their resistance within 30 min in interrupted matings with 5199, and 2-h uninterrupted matings with JC1552 suggested that these resistance genes were located between *metB* and *mtl*. As the genes conferring NAL resistance around there have not been reported, the resistance gene of N-21 and N-31 was designated as *nalC*, and that of N-24 was designated as *nalD*. To locate *nalC* and *D* more precisely, P1 transduction was carried out by using AB3505 as a recipient (Table 1). NAL resistance was not detected

when selected for *metE*$^+$. However, *nalC* of N-21 and N-31, and *nalD* of N-24 were cotransducible with *ilvD* at frequencies of 16.4 and 8.3% (*ilvD*$^+$ and *nal*^{r} selections), 12.4 and 20%, and 10.2 and 7.1%, respectively. All but two of the *ilvD*$^+$ *nalC*^{r} cotransductants and all of the *ilvD*$^+$ *nalD*^{r} cotransductants were *metE*, indicating that *nalC* and *D* were situated near *ilvD* on the side opposite to *metE*. All of the NAL-resistant transductants tested had the same patterns of resistance to PPA, PA, and NAL as the donors. By applying cotransduction frequencies in *ilvD*$^+$ selection to Wu's formula (17), the distances from *ilvD* (83 min) to *nalC* and *D* can be calculated to be about 0.9 to 1.0 min and 1.1 min, respectively. Therefore, *nalC* and *D* would be located at approximately 82 min on the map. Trials for isolating *nalC nalD* double mutants by P1 transduction remain unsuccessful up to now.

Inhibition of DNA synthesis. The levels of resistance to PPA, PA, and NAL in the DNA synthesis of the mutants were examined with or without EDTA treatment (Table 2). In KL-16, the ID_{50} values of PPA, PA, and NAL were 3.0, 7.0, and 2.5 μg/ml in intact cells, and 1.3, 0.3, and 0.6 μg/ml in EDTA-treated cells, the ID_{50} ratios being 2.3, 23.3, and 4.2, respectively. In the *nalA* mutants, N-51, P-18, and P-5, the ID_{50} values of PPA, PA, and NAL were 25 to 50 μg/ml, 75 to more than 400 μg/ml, and 29 to 400 μg/ml, respectively, in intact cells, and 12 to 23 μg/ml,

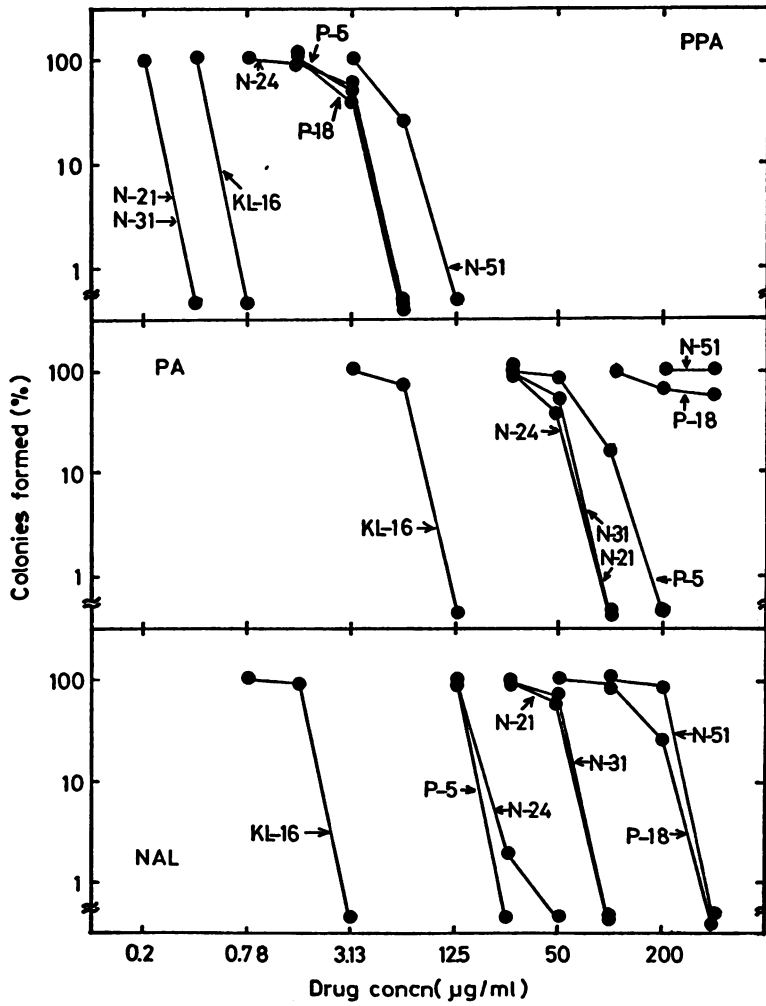

FIG. 2. *Colony-forming ability of spontaneous mutants of E. coli KL-16 resistant to PPA, PA, and NAL in the presence of the drugs. N-21, N-24, N-31, and N-51 were selected with NAL, and P-5 and P-18 were selected with PPA.*

TABLE 1. *P1 transduction of nal genes using N-21, N-31, and N-24 as donors and AB3505 as a recipient*[a]

Selected marker	Unselected marker	N-21		N-31		N-24	
		No.[b]	%	No.	%	No.	%
metE$^+$	*ilvD*$^-$ *nal*^{s}	30/129	23.3	24/120	20.0	31/119	26.1
	ilvD$^-$ *nal*^{r}	0/129	0	0/120	0	0/119	0
	ilvD$^+$ *nal*^{s}	99/129	76.7	96/120	80.0	88/119	73.9
	ilvD$^+$ *nal*^{r}	0/129	0	0/120	0	0/119	0
ilvD$^+$	*metE*$^-$ *nal*^{r}	218/299	72.9	136/178	76.4	239/294	81.3
	metE$^-$ *nal*^{r}	49/299	16.4	22/178	12.4	30/294	10.2
	metE$^+$ *nal*^{s}	31/299	10.4	19/178	10.7	25/294	8.5
	metE$^+$ *nal*^{r}	1/299	0.3	1/178	0.6	0/294	0
nal^r	*metE*$^-$ *ilvD*$^-$	33/36	91.7	12/15	80.0	13/14	92.9
	metE$^-$ *ilvD*$^+$	3/36	8.3	3/15	20.0	1/14	7.1
	metE$^+$ *ilvD*$^-$	0/36	0	0/15	0	0/14	0
	metE$^+$ *ilvD*$^+$	0/36	0	0/15	0	0/14	0

[a] Relevant markers: N-21, *metE*$^+$ *ilvD*$^+$ *nalC*^{r}; N-31, *metE*$^+$ *ilvD*$^+$ *nalC*^{r}; N-24, *metE*$^+$ *ilvD*$^+$ *nalD*^{r}; and AB3505, *metE ilvD nalC*^{s} *nalD*^{s}.

[b] Number of transductants having indicated unselected markers/number of transductants tested.

TABLE 2. *Levels of resistance to PPA, PA, and NAL in the DNA synthesis of spontaneous mutants resistant to the drugs*

Mutated gene	Strain	ID_{50}[a] of PPA			ID_{50} of PA			ID_{50} of NAL		
		Intact[b]	EDTA[c]	Intact/EDTA[d]	Intact	EDTA	Intact/EDTA	Intact	EDTA	Intact/EDTA
	KL-16	3.0	1.3	2.3	7.0	0.3	23.3	2.5	0.6	4.2
nalA	N-51	45	20	2.3	>400	30	>13.3	400	100	4.0
	P-18	50	23	2.2	>400	27	>14.8	300	100	3.0
	P-5	25	12	2.2	75	3.5	21.0	29	8.6	3.4
nalC	N-21	0.3	0.2	1.5	42	1.1	38.2	60	9.5	6.3
	N-31	0.5	0.4	1.3	34	1.1	30.9	61	10	6.1
nalD	N-24	8.4	4.0	2.1	76	0.9	84.5	52	4.5	11.6

[a] ID_{50} of DNA synthesis in micrograms per milliliter.

[b] Intact cells.

[c] EDTA-treated cells.

[d] ID_{50} ratio (intact cells/EDTA-treated cells).

3.5 to 30 μg/ml, and 8.6 to 100 μg/ml in EDTA-treated cells. Their ID_{50} ratios were almost the same as those in KL-16. The levels of resistance to PPA were significantly lower than those to PA and NAL in intact cells of N-51 and P-18. In the *nalC* mutants, N-21 and N-31, the ID_{50} values of PA and NAL were 34 to 42 μg/ml and 60 to 61 μg/ml, respectively, in intact cells, and 1.1 μg/ml and 9.5 to 10 μg/ml in EDTA-treated cells. However, those of PPA were 0.3 to 0.5 μg/ml in intact cells, and 0.2 to 0.4 μg/ml in EDTA-treated cells. Their ID_{50} ratios for PA and NAL were slightly higher than those in KL-16, whereas those for PPA were slightly lower. Resistance to PA and NAL and hypersusceptibility to PPA were thus observed in the level of DNA synthesis. In the *nalD* mutant, N-24, the ID_{50} values of PPA, PA, and NAL were 8.4, 76, and 52 μg/ml, respectively, in intact cells, and 4.0, 0.9, and 4.5 μg/ml in EDTA-treated cells. Its ID_{50} ratio for PPA was nearly equal to that in KL-16, but that for PA and NAL was about three times higher. PPA was significantly different from PA and NAL in the ID_{50} ratio of this mutant.

DISCUSSION

PPA is a synthetic antibacterial agent structurally related to PA and NAL. One of the interesting properties of PPA is that most clinically isolated bacteria highly resistant to PA and NAL are susceptible to PPA to some extent. To study on this incomplete cross-resistance, spontaneous mutants resistant to PPA, PA, and NAL were isolated from *E. coli* KL-16. Their levels of resistance to PPA were usually lower than those to PA and NAL. Some mutants were found to

be resistant to PA and NAL but hypersusceptible to PPA.

Genetic studies with several mutants with relatively high-level resistance revealed that resistance was conferred by mutations at *nalA*, and new *nal* genes designated as *nalC* and *D*. The *nalA* and *D* mutations conferred resistance to PPA, PA, and NAL, whereas the *nalC* mutations determined resistance to only the latter two drugs. Mutants with low-level resistance which might be governed by *nalB* (7) were not used in the present study. The *nalC* and *D* were mapped at about 82 min on the recalibrated linkage map of *E. coli* K-12. Around this region, the *cou* gene determining resistance to coumermycin and novobiocin, whose product is a component of DNA gyrase (5), as well as the *nalA* gene product (4, 16), has been mapped (13). However, the *nalC* or *D* mutants were no more resistant to novobiocin than KL-16 (data not shown). As trials for isolating *nalC nalD* double mutants by P1 transduction so far made are not successful, it is not obvious whether *nalC* and *D* are different genes close to each other or a single gene with different phenotypes.

When DNA synthesis was examined in intact and EDTA-treated cells, the *nalA* mutants were resistant to all the three drugs in both the cell systems. In EDTA-treated cells, the levels of resistance were always lower than those in intact cells, the degrees of lowering depending on the drugs tested. The degrees of EDTA sensitization were the same in the *nalA* mutants as in KL-16. If the experimental conditions used for EDTA-treatment broke the permeability barrier of *E. coli* as shown by Leive (8), the result would mean that the *nalA* mutants are not transport mutants but mutants with decreased sensitivity of the DNA synthesizing system to the drugs. The same result has been reported by Bourguignon (2). The levels of resistance to PPA were usually lower than those to PA and NAL in the intact cells of the *nalA* mutants.

The *nalC* mutants showed resistance to PA and NAL but hypersensitivity to PPA in the DNA synthesis of both the cell systems with a little change in the degree of EDTA sensitization. So, *nalC* is considered to be a gene determining sensitivity of the DNA synthesizing system to the drugs.

The *nalD* mutant was resistant to all three drugs in the DNA synthesis of intact and EDTA-treated cells, but the degrees of EDTA sensitization were significantly marked compared with KL-16 only for PA and NAL. This result suggests that *nalD* is a gene determining sensitivity of the DNA synthesizing system to PPA, PA, and NAL and at the same time decreased permeability to PA and NAL but not to PPA. It is unlikely that the *nalD* mutant is a double mutant with a mutation on the DNA synthesizing system and a mutation on drug transport, for similar strains to the *nalD* mutant have been found among relatively small number of spontaneously isolated mutants tested (data not shown) and lowering in resistance to PA and NAL was not observed in the *nalD*^{r} transductants tested so far. However, it is not easy to understand that a single *nalD* mutation causes a simultaneous change of drug sensitivity of the DNA synthesizing system and drug transport unless the permeability barrier is in some way related to the DNA synthesizing system. It may be possible that a structural change of the DNA synthesizing system on the cell membrane due to a mutational change of its components affects permeability to the drugs through the membrane.

As for the mutants tested, it is concluded that incomplete cross-resistance among PPA, PA, and NAL would be ascribed to differences in drug sensitivity of the bacterial DNA synthesizing system and in drug permeability.

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