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New Nalidixic Acid Resistance Mutations Related to Deoxyribonucleic Acid Gyrase Activity

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In *Escherichia coli* K-12 mutants which had a new nalidixic acid resistance mutation at about 82 min on the chromosome map, cell growth was resistant to or hypersusceptible to nalidixic acid, oxolinic acid, piromidic acid, pipemidic acid, and novobiocin. Deoxyribonucleic acid gyrase activity as tested by supercoiling of λ phage deoxyribonucleic acid inside the mutants was similarly resistant or hypersusceptible to the compounds. The drug concentrations required for gyrase inhibition were much higher than those for cell growth inhibition but similar to those for inhibition of λ phage multiplication. Transduction analysis with λ phages carrying the chromosomal fragment of the *tnaA-gyrB* region suggested that one of the mutations, *nal-31*, was located on the *gyrB* gene.

Nalidixic acid (NA) (27), oxolinic acid (OA) (24), piromidic acid (PA) (34), and pipemidic acid (PPA) (29) are synthetic antibacterial compounds having a pyridonecarboxylic acid moiety as a common chemical structure and are mainly active against gram-negative bacteria (9, 43, 45, 46). These compounds specifically inhibit bacterial DNA synthesis with little effect on RNA and protein synthesis around their minimal inhibitory concentrations (6, 43). The inhibition of DNA synthesis is so rapid that the compounds are considered to act during the elongation of DNA chains (6, 10).

Recently, two research groups found that NA and OA inhibited an enzyme called DNA gyrase (15, 50). This enzyme introduces negative superhelical turns into duplex DNA (16) and is considered to play an important role in metabolic processes involving DNA (7, 25, 38). Gyrase from *Escherichia coli* consists of two subunits, A and B (20). Subunit A is a homodimer of a 105,000-dalton protein encoded by the *gyrA* (formerly *nalA*) gene, which determines resistance to NA, and subunit B consists of a 95,000-dalton protein encoded by the *gyrB* (formerly *cou*) gene, which determines resistance to novobiocin (NB) and coumermycin A_1 (20, 35). Subunit A from an NA-resistant *gyrA* mutant and subunit B from a coumermycin A_1-resistant *gyrB* mutant each reconstitute a gyrase with the expected drug resistance. The *gyrA* gene is located at approximately 48 min, and the *gyrB* gene is at approximately 82 min on the genetic map of *Escherichia coli* K-12 (2).

Cross-resistance is usually observed among pyridonecarboxylic acid derivatives, but it is so incomplete that bacteria which are highly resistant to NA are inhibited by some of them, e.g., PPA, at relatively low concentrations (46). During a study on the mode of incomplete cross-resistance, two new types of NA resistance mutations were found at about 82 min, i.e., *nal-21* and *nal-31* (formerly *nalC*), and *nal-24* (formerly *nalD*) (22). The *nal-21* or *nal-31* mutation caused resistance to NA and PA but also hypersusceptibility to PPA. The *nal-24* mutation, like NA resistance *gyrA* mutations, conferred resistance to all three drugs. The *nal-21*, *nal-31*, and *nal-24* mutations were no more resistant to NB by a conventional agar dilution method for the determination of minimal inhibitory concentrations, but were twice as resistant by the colony-former reduction assay. The *nal-21* and *nal-31* mutations affect sensitivity of the DNA synthesizing system to the compounds, and the *nal-24* mutation affects both sensitivity of the DNA synthesizing system and drug transport (22).

Recently, we found that the *nal-31* and *nal-24* mutations caused changes in drug susceptibility of in vivo gyrase activity in the same direction as drug susceptibility of the growth or DNA synthesis of the mutants. The mutational site of *nal-31*, but not that of *nal-24*, probably resides on a part of the *gyrB* gene carried by a *tnaA dnaA recF* transducing phage, λ *dnaA4*.

MATERIALS AND METHODS

Bacteria and phages. All of the bacteria used were derivatives of *E. coli* K-12. The derivation of strain KL-16 (Hfr, *thi relA*), its spontaneous NA resistance mutants N-51 *gyrA-51*, N-31 *nal-31*, and N-24 *nal-24*, and P1 *kc* was described previously (22). Strain LC257 (F^- *dnaA46 thy leu* λ^s) was obtained from H. Ryo; Km1012 (F^- *gal thy* λ *cI857 S7*) and QD5003 (F^-

mel SuIII) were from K. Matsubara; strain 153 (F^- *tnaA* Δ[*trpA-E-tonB*] λ^-) and *dnaA*-transducing λ phages (λ *tna*, λ *dnaA*4 Ap52, and λ *dnaA*4) were from H. Yamagishi. The *nal-31* and *nal-24* derivatives of strain 153 were made by P1 transduction by the method of Miller (32). λ lysogens of strain KL-16 and its mutants were prepared by the method of Tomizawa (51).

Chemicals. The pyridonecarboxylic acid derivatives used were synthesized in this laboratory (24, 27, 29, 33). Equimolar NaOH was added to dissolve the compounds in water. Sodium ampicillin was purchased from Meiji Seika Kaisha, Ltd.; sodium NB, lysozyme (grade I), DNase I (DN-CL), and RNase A (type I-A) were from Sigma Chemical Co.; phenol (redistilled nucleic acid grade) was from Bethesda Research Laboratories, Inc.; agarose (ME) was from Marine Colloids, Inc.; [*methyl*-^{14}C]thymidine (55 mCi/mmol) was from The Radiochemical Centre; other reagents (guaranteed reagent) were from Nakarai Chemicals, Ltd.

Media and buffers. Casamino Acids λ medium or agar (21), λ broth or agar (52), Tna medium for Tna^+ selection (31), R-top agar, LB medium or agar, and minimal medium (22) were prepared as described previously. Tris dilution buffer (21) and a 10 mM $MgSO_4$ solution were used for stock and for dilution of λ phages, and TE buffer (1 mM disodium EDTA, 10 mM Tris-hydrochloride, pH 7.6) was used to dissolve the DNA. Supplements were added, when necessary, at the following concentrations (micrograms per milliliter): L-leucine, 20; thiamine hydrochloride, 1; and thymine, 50.

Measurement of drug susceptibility. To check drug susceptibility of the bacterial strains, the colony-former reduction assay was employed. About 300 cells per plate were mixed with 10 ml of Casamino Acids λ agar containing the drugs at graded concentrations and incubated at 37°C overnight, and the number of colonies formed was counted.

Preparation of λ phage with ^{14}C-labeled DNA. *E. coli* Km1012 (*gal thy* λ c1857*S*7) grown exponentially at 30°C in Casamino Acids λ broth (pH 7.0) was harvested by centrifugation and resuspended in 0.1 volume of the same broth (about 5×10^9 cells per ml). The suspension was kept at 43°C for 20 min to inactivate the temperature-sensitive λ cI repressor. Immediately after heat treatment, [*methyl*-^{14}C]thymidine (55 mCi/mmol) was added to the suspension at a final concentration of 2.5 μCi/ml, and this mixture was then incubated for 3 h at 37°C with shaking. After incubation, the cells were lysed with chloroform and then centrifuged. The supernatant was digested with DNase A and RNase A by the method of Hirose (21), and λ phage containing ^{14}C-labeled DNA was pelleted by ultracentrifugation at 64,500 × *g* for 2 h at 4°C. The pellet was suspended in Tris dilution buffer and stored at 4°C until use.

Assay of DNA gyrase activity. DNA gyrase activity was assayed by the supercoiling activity of λ phage in vivo. The assay condition was the same as that described by Gellert et al. (15, 17) except for a few modifications. The cultures of the λ lysogenic strains grown at 37°C to a density of approximately 5×10^8 cells per ml in Casamino Acids λ broth (pH 7.5) containing 10 mM $MgSO_4$ were divided into 360-μl portions, incubated for 5 min at 37°C in the presence or absence of drugs, and superinfected with λ c1857*S*7 phage having ^{14}C-labeled DNA at a multiplicity of five. After incubation for 15 min at 37°C under static conditions, the infected cells were chilled, washed once with the same broth, and suspended in 100 μl of 25% sucrose in 50 mM Tris buffer, pH 8.0. Cleared lysates were prepared by the method of Guerry et al. (18). The lysates were digested with RNase A, extracted with phenol, and mixed with twice the volume of cold isopropyl alcohol to precipitate DNA as described by Hirose (21) and Meyers et al. (30). Precipitated DNA was centrifuged at 10,000 × *g* at −10°C for 20 min and dissolved in 50 μl of TE buffer. The DNA samples were analyzed by 0.3% agarose gel electrophoresis by the method of Aaij and Borst (1), using a slab disc gel apparatus (SJ-1060.SDH type, Mitsumi Scientific Industry Co., Ltd.). After electrophoresis at 50 V for about 5 h, the gel was removed from the apparatus, fixed overnight with 10% trichloroacetic acid, and processed for fluorography as described by Laskey et al. (3, 26, 39). After exposure for 7 days, the density of the bands of relaxed and supercoiled λ DNA on X-ray film was scanned with a digital computing densitometer (DCD-16 type, Gelman Instruments Co.). The percentage of supercoiled λ DNA was calculated from the following formula: 100 × (area under the density peak of supercoiled DNA fraction of drug-treated sample)/(area under the density peak of supercoiled DNA fraction of control).

Measurement of inhibition of phage growth. *E. coli* (λ^+) cells grown exponentially in λ broth were harvested by centrifugation, washed once with 0.1 M $MgSO_4$ solution, and resuspended in the same solution (about 2×10^8 cells per ml). A 5-ml amount of the suspension was spread on glass petri dishes, irradiated with UV light (110 ergs/mm^2), immediately mixed with 5.0 ml of the prewarmed λ broth, and incubated in the dark at 37°C for 2.5 h with shaking in the presence or absence of drugs. After incubation, chloroform was added, and cell debris was eliminated by centrifugation. The supernatant was assayed for plaque-forming units on *E. coli* KL-16.

Transduction analysis. P1 transduction was performed as described previously (22). Specialized transduction by transducing λ phages was carried out as follows. An *nal-31* or *nal-24* derivative of *E. coli* 153 was grown in LB broth containing 10 mM $MgSO_4$ at a density of about 10^9 cells per ml, infected with transducing λ phages at a multiplicity of about 0.1, incubated at 37°C for 20 min for adsorption, diluted 10 times with the same broth, and cultured at 37°C for 2 h for expression. A 0.1-ml amount of the infected cultures was spread over the surface of selective agar plates (Tna medium plates and LB agar plates containing PPA at 0.78 μg/ml or ampicillin at 50 μg/ml). After incubation at 37°C overnight, the colonies formed were checked for unselected markers by the method described previously (22).

RESULTS

Drug susceptibility of the strains used. Drug susceptibility of *E. coli* strains used in this study was examined by checking their colony-forming ability in the presence of the drugs at graded concentrations (Table 1). In the wild-

TABLE 1. *Susceptibility of E. coli K-12 derivatives to pyridonecarboxylic acids and NB*

Strain	Relevant genotype	Inhibitory concn (μg/ml)[a]				
		PPA	PA	NA	OA	NB
KL-16	wild type	1.56	12.5	3.13	0.2	50
N-51	*gyrA-51*	12.5	>400	400	6.25	50
N-31	*nal-31*	0.39	100	100	3.13	100
N-24	*nal-24*	6.25	100	50	0.78	100

[a] Minimum drug concentration inhibiting colony formation by more than 99%.

type strain, KL-16, the colonies formed were reduced by more than 99% with 1.56 μg of PPA per ml, 12.5 μg of PA per ml, 3.13 μg of NA per ml, 0.2 μg of OA per ml, and 50 μg of NB per ml. The *gyrA-51* mutant, N-51, was 8, >32, 128, and 32 times more resistant to PPA, PA, NA, and OA, respectively, than was strain KL-16, but no more resistant to NB. The *nal-31* mutant, N-31, was 8, 32, and 16 times more resistant to PA, NA, and OA, respectively, but 4 times more susceptible to PPA than was strain KL-16. The *nal-24* mutant, N-24, showed a similar resistance pattern to N-51 but was lower in the level of resistance than N-51. N-31 and N-24 were two times more resistant to NB than was KL-16 in this experiment. Drug susceptibility of the strains was unaffected by λ lysogenization (data not shown).

Inhibition of DNA gyrase activity in vivo. When phage λ superinfects *E. coli* cells already lysogenic for λ, most of the superinfecting λ DNA is converted to closed circular relaxed DNA by DNA ligase and then changed to supercoiled DNA by the action of DNA gyrase (56), but the replication of phage DNA is inhibited by the λ cI repressor. Taking advantage of this supercoiling reaction, drug susceptibility of gyrase activity in vivo was examined in strain KL-16 and its *nal* mutants. Figure 1 shows susceptibility of gyrase activity in strain KL-16 to pipemidic acid. The supercoiled fraction of λ DNA decreased with rising PPA concentration and the relaxed fraction was inversely proportionate to the supercoiled fraction. In strain KL-16 (Fig. 2A), the gyrase 50% inhibitory dose (ID_{50}) values at which supercoiled λ DNA was reduced to 50% of that of the control were 21, 54, 78, and 420 μg/ml for OA, PPA, NA, and PA, respectively. A higher antibacterial activity was paralleled by a higher antigyrase activity, although the concentrations required for gyrase inhibition were about one or two orders of magnitude higher than those for inhibition of colony formation. The ID_{50} value of NB was 170 μg/ml, which was low considering its relatively weak antibacterial activity. In strain N-51 (Fig. 2B), the ID_{50} value of PPA was 1,350 μg/ml, whereas gyrase inhibition was only 5 to 20% at the maximum concentrations of PA, NA, and OA, i.e., 1,600, 1,600 and 400 μg/ml, respectively. The ID_{50} value of NB was 150 μg/ml, which was almost the same as that for strain KL-16. In strain N-31 (Fig. 2C), gyrase activity was highly resistant to OA, NA, and PA, whereas it was more susceptible to PPA than was that in strain KL-16. Thus, the unique resistance pattern of the *nal-31* mutation in antibacterial activity was also observed in gyrase inhibition. The ID_{50} value of NB, 300 μg/ml, was about two times higher than that in strain KL-16. In strain N-24 (Fig. 2D), the ID_{50} values of OA, PPA, NA, and PA were significantly higher than those in strain KL-16, and that of NB was slightly higher than that of KL-16. These results indicated that *nal-31* and *nal-24* as well as *gyrA-51* are mutations in the genes relating to gyrase activity in vivo and that the inhibitory effects of pyridonecarboxylic acid derivatives and NB on gyrase activity in vivo are correlated to their antibacterial activities. However, the concentrations required

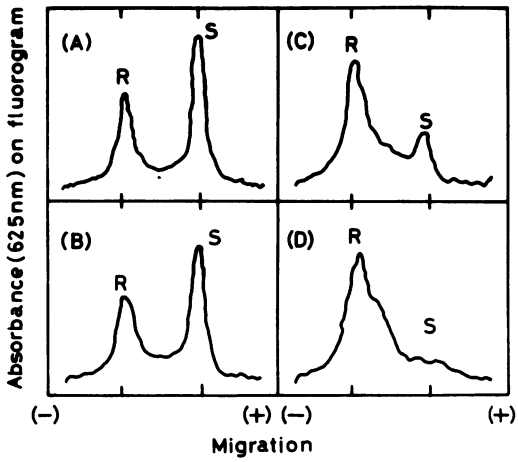

FIG. 1. *Electrophoretic profiles of relaxed and supercoiled λ DNA made in vivo under the effect of PPA. E. coli KL-16 (λ) was superinfected with λ cI857S7 having [^{14}C]DNA in the absence (A) or presence of PPA (B, 25 μg/ml; C, 50 μg/ml; and D, 100 μg/ml). Supercoiled (S) and relaxed (R) λ DNAs were analyzed by agarose gel electrophoresis, fluorography, and densitometry.*

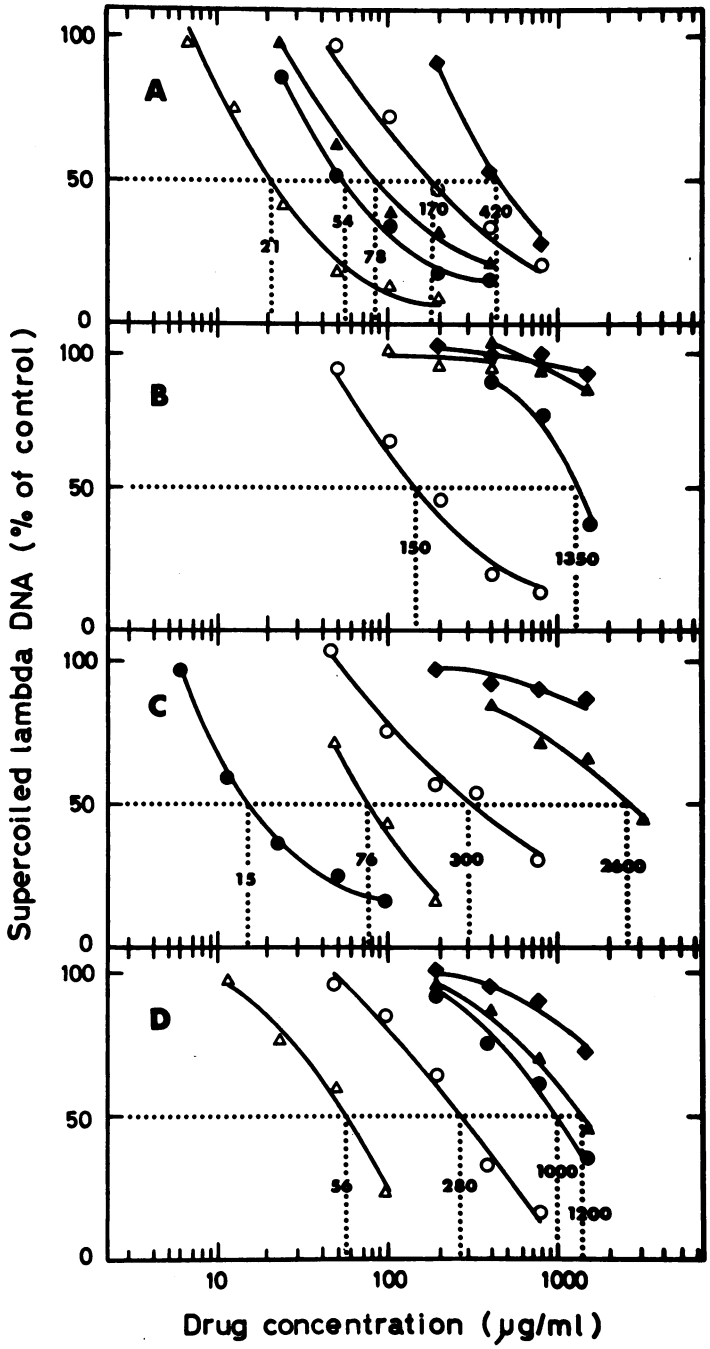

FIG. 2. *Susceptibility of gyrase activity in vivo to pyridonecarboxylic acids and NB. The strains used were the λ lysogens of E. coli strains KL-16 (A), N-51 (B), N-31 (C), and N-24 (D). ID_{50} values (dotted lines) are as indicated. Symbols: PPA, ●; PA, ◆; NA, ▲; OA, △; NB, ○.*

for gyrase inhibition were much higher than those required for growth inhibition, especially with the pyridonecarboxylic acid derivatives.

Inhibition of the growth of phage λ. The effect of pyridonecarboxylic acid derivatives and NB on the growth of phage λ was investigated

to elucidate the high drug concentrations for gyrase inhibition. When phage λ was grown in strain KL-16, the plaques formed were reduced by more than 99%, with 12.5 μg of OA per ml, 50 μg of PPA per ml, 100 μg of NA per ml, 800 μg of PA per ml, and 200 μg of NB per ml (Fig. 3). These concentrations were similar to those required for gyrase inhibition and were in great contrast to those for the inhibition of cell growth (Table 1). The result suggested that drug susceptibility of gyrase activity in vivo may depend on the DNA replicating systems employed.

Transduction analysis. As described previously (22), *nal-31* and *nal-24* are situated at approximately 82 min on the *E. coli* K-12 map. To determine the precise loci of the mutations, P1 transduction was carried out by using the temperature-sensitive *dnaA* mutant LC257 as a recipient and *nal* mutants N-31 and N-24 as donors. When selected for either *dnaA*$^+$ or *nal*, *nal-31* and *nal-24* were cotransducible with *dnaA*$^+$ at frequencies of 100%, irrespective of the selected markers (Table 2). This suggests that the *nal* genes were situated very close to the *dnaA* gene. In the vicinity of *dnaA* gene, several genes are considered to exist in the clockwise order of *uhp*, *gyrB*, *recF*, *dnaA*, *rimA*, *tnaA*, and *bglB* (2, 19, 36). A *tnaA dnaA recF* transducing λ phage, λ *dnaA*4, is known to carry a wild-type chromosomal DNA fragment of the *tnaA-gyrB* region of 13.8 kilobase pairs long, substituting for phage genes from *int* to *gam* (36). When a *nal-31* derivative of strain 153, 153-4 *tnaA nal-31*, was infected with λ *dnaA*4 and selected for Tna$^+$, 0.8% of Tna$^+$ colonies were Nal-31$^+$, 89.3% were mixtures of a large number of Nal-31 cells and a small or sometimes equal number of Nal-31$^+$ cells, and 9.9% were Nal-31 (Table 3). Nal-31 cells, but not Nal-31$^+$ cells, purified from the second type of colonies formed the same mixture of colonies upon retesting, suggesting that Nal-31$^+$ cells were segregated from Nal-31 cells. However, Nal-31$^+$ cells of the first type and Nal-31 cells of the third type gave the same results upon retesting after colony purification, suggesting that they were genetically stable. On the other hand, all of the colonies selected for Nal-31$^+$ were Tna$^+$ and genetically stable. A derivative of λ *dnaA*4 deleting *dnaA* and *recF*, λ *dnaA*4-Ap52, is considered to bear 4.3- and 2.6-kilobase-pair chromosomal DNA fragments including the *tnaA* gene and probably a part of the *gyrB* gene, respectively (36). Nal-31$^+$ transductants were similarly ob-

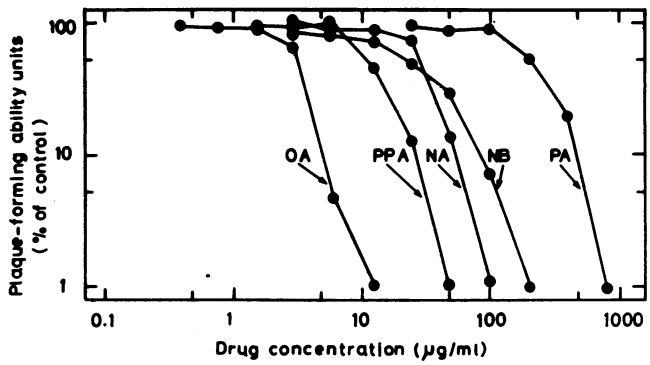

FIG. 3. *Effect of pyridonecarboxylic acids and NB on the growth of phage* λ.

TABLE 2. *P1 transduction of nal genes with E. coli N-31 and N-24 as donors and LC257 as a recipient*[a]

Selected markers	Unselected markers	N-31		N-24	
		No.[b]	%	No.	%
DnaA$^+$	Nal$^+$ IlvD$^+$	0/106	0	0/110	0
	Nal IlvD$^+$	104/106	98.1	107/110	97.3
	Nal IlvD	2/106	1.9	3/110	2.7
	Nal$^+$ IlvD	0/106	0	0/110	0
Nal	DnaA IlvD$^+$	0/109	0	0/109	0
	DnaA$^+$ IlvD$^+$	106/109	97.2	107/109	98.2
	DnaA$^+$ IlvD	3/109	2.8	2/109	1.8
	DnaA IlvD	0/109	0	0/109	0

[a] Relevant markers: N-31, *ilvD nal-31*; N-24, *ilvD nal-24*; and LC257, *ilvD*$^+$ *nal-31*$^+$ *nal-24*$^+$ *dnaA46*.
[b] Number of transductants having indicated unselected markers/number of transductants tested.

TABLE 3. *Specialized transduction of tnaA⁺ and Nal⁺ genes by λ dnaA4, λ dnaA4-Ap52, and λ tna*

Recipient cells	Transducing phage	Selected markers	Unselected markers	Transductants	
				No.[a]	%
E. coli 153-4 (*tnaA nal-31*)	λ *dnaA4* (*tnaA⁺ dnaA⁺ recF⁺*)	Tna⁺	Nal-31⁺	5/616	0.8
			Nal-31 (Nal-31⁺)[b]	550/616	89.3
			Nal-31	61/616	9.9
		Nal-31⁺[c]	Tna⁺	114/114	100
			Tna⁻	0/114	0
	λ *dnaA4*-Ap52 (*tnaA⁺ amp^r*)	Tna⁺	Nal-31⁺ Amp^r	4/112	3.6
			Nal-31 (Nal-31⁺) Amp^r	77/112	68.8
			Nal-31 Amp^r	5/112	4.5
			Nal-31 Amp^s	26/112	23.2
		Amp^r	Nal-31⁺ Tna⁺	0/112	0
			Nal-31 (Nal-31⁺) Tna⁺	79/112	70.5
			Nal-31 Tna⁺	13/112	11.6
			Nal-31 Tna⁻	20/112	17.9
		Nal-31⁺	Tna⁺ Amp^r	133/133	100
			Tna⁻ Amp^r or s	0/133	0
	λ *tna* (*tnaA⁺*)	Tna⁺	Nal-31⁺	0/120	0
			Nal-31	120/120	100
E. coli 153-1 (*tnaA nal-24*)	λ *dnaA4* (*tnaA⁺ dnaA⁺ recF⁺*)	Tna⁺	Nal-24⁺	0/660	0
			Nal-24	660/660	100

[a] See Table 2, footnote *b*.

[b] Purified Tna⁺ colonies were mixtures of a large number of Nal-31 cells and a small or sometimes equal number of Nal-31⁺ cells.

[c] Nal-31⁺ colonies were selected on PPA (0.78 μg/ml) plates. Five percent of the PPA-resistant colonies showed the GyrA phenotype and were excluded from calculation.

tained by infecting strain 153-4 with λ *dnaA4*-Ap52. However, no Nal-31⁺ transductants were detected in the infection with λ *tna*, which carries only a 6.2-kilobase-pair chromosomal fragment including the *tnaA* gene (36). These results demonstrate that the *nal-31* is a mutation probably on the DNA fragment including a part of the *gyrB* gene carried by λ *dnaA4* and λ *dnaA4*-Ap52. When a *nal*-24 derivative of strain 153, 153-1 *tnaA nal-24*, was infected with λ *dnaA4* and selected for Tna⁺, none of the Tna⁺ colonies were *nal-24⁺*. These results suggest that the mutational site for *nal-24* does not reside on the chromosomal region carried by λ *dnaA4*.

DISCUSSION

DNA gyrase [*Eco* DNA topoisomerase II] has at least six activities; DNA supercoiling, binding to DNA, DNA relaxation, breakage reunion of DNA, ATPase, and catenation or uncatenation of circular DNA (7). This enzyme is required for λ integrative recombination (34), transcription (44, 47, 52), and DNA replication of *E. coli* (8), plasmids (42), and bacteriophages (23, 28, 46). Although the precise physiological role of gyrase remains unclear, there is good evidence that gyrase is responsible for introducing and maintaining the supercoiling of DNA in vivo (11, 50), which is considered to be important for the interaction of DNA and enzymes participating in metabolic processes involving DNA (7, 38). As temperature-sensitive mutants of *gyrA* or *gyrB* are lethal at high temperature (25, 37), gyrase is considered to be essential for the growth of *E. coli*.

The present study showed that PPA and PA inhibited gyrase activity in vivo as NA and OA did. This result suggests that the inhibition might be common to many of this group of compounds. Compounds with higher antibacterial activity showed potent anti-gyrase activity. However, concentrations of the compounds needed for gyrase inhibition were much higher than concentrations needed for growth inhibition. Gellert et al. reported a similar result (15). They stated that the cells either must be sensitive to very small changes in DNA supercoiling, or more plausibly, some more subtle interaction of gyrase or of subunit A is involved for cell growth. The former idea is inconsistent with the fact that PA, which is less potent in gyrase inhibition than NB, is more potent in the inhibition of cell growth. On the other hand, the multiplication of λ phage was inhibited by pyridonecarboxylic acid derivatives and NB at con-

centrations very similar to those for gyrase inhibition. This result suggests that higher concentrations for gyrase inhibition may be due to employing λ DNA instead of chromosomal DNA as a substrate for gyrase activity in vivo. It is noteworthy that the breakage of chromosomal DNA probably caused by gyrase or related enzymes in the presence of OA is observable around its growth inhibitory concentration (48).

A possible interpretation is that the structures and the sites of the DNA-synthesizing apparatus of chromosomal and λ DNAs are different from each other, and gyrase in the latter apparatus is relatively inaccessible for drugs. Partition coefficients of PPA, PA, NA, OA, and NB between *n*-octanol and 0.01 M phosphate buffer, pH 7.2, were −1.688, 0.687, 0.193, 0.272, and 1.31, respectively. High lipophylicity of NB compared with that of pyridonecarboxylic acid derivatives could account for its high antigyrase and its low antibacterial activities, provided that the λ DNA synthesizing apparatus is surrounded by lipophylic circumstances.

An alternative interpretation may be that cell growth but not λ phage growth requires an enzyme other than gyrase which is inhibited by pyridonecarboxylic acid derivatives. A possible candidate is *Eco* topoisomerase II′ (4, 14). It consists of gyrase subunit A and a 50,000-dalton protein called *ν* (4), which is supposed to be a processed form of gyrase subunit B or derived from a transcript of part of *gyrB* (4, 16). *Eco* topoisomerase II′ does not supercoil but relaxes DNA and is unaffected by either ATP or NB. The enzyme closely resembles gyrase in other properties including sensitivity to OA, stabilization of positive supercoils, double-strand cleavage at the same DNA sites as gyrase, catenation, and uncatenation of DNA rings (7). Although the role of topoisomerase II′ in chromosome replication is not clear, it is suggestive that T4 topoisomerase, which is similar to topoisomerase II′ with respect to lacking supercoiling activity and possessing positive supercoil-relaxing activity (4), seems to be required for proper initiation of T4 DNA replication (7).

The present study also showed that gyrase activity in vivo was resistant or hypersusceptible to pyridonecarboxylic acid derivatives and weakly resistant to NB in the *nal-31* and *nal-24* mutants. This result suggests that *nal-31* and *nal-24* are mutations on the gene relating to gyrase activity in vivo. P1 transduction showed that the mutations were situated very close to *dnaA*. Transduction experiments with transducing λ phages carrying the *tnaA-gyrB* region revealed that the *nal-31* mutation was on a 2.6-kilobase-pair chromosomal fragment carried by λ *dnaA*4-Ap52. The 2.6-kilobase-pair fragment has been known to code for a protein of approximately 72,000 daltons which is supposed to be the N terminus of the gyrase subunit B protomer (95,000 daltons) (H. Yamagishi, personal communication). This fact and the size of the DNA fragment indicate that the *gyrB* gene carried by the phages is deleted in part. As the *nal-31*$^+$ transducing phages λ *dnaA*4 and λ *dnaA*4-Ap52 had lost the *int* gene, the DNA carried by the phages was usually integrated as a whole into the *tnaA-gyrB* region of hosts through reciprocal recombination between the homologous regions commonly carried by the phages and the host chromosome. When strain 153-4 *tnaA nal-31* was infected with λ *dnaA*4 or λ *dnaA*4-Ap52, most of the colonies selected for Tna$^+$ showed the Nal-31 phenotype and segregated a small number of Nal-31$^+$ progenies, but all of the colonies selected for Nal-31$^+$, which were about two orders of magnitude less than those selected for Tna$^+$, were Tna$^+$ and genetically stable. This result suggests that phage infection per se is sufficient for the Tna$^+$ phenotype but the formation of the complete wild-type *gyrB* gene through recombination is necessary for the Nal-31$^+$ phenotype. On the other hand, the colonies with the Nal-24$^+$ phenotypes were not detected upon infection of strain 153-1 *tnaA nal-24* with λ *dnaA*4. These results indicate that the *nal-31* mutation resides on a phage-carried part of the *gyrB* gene and the *nal-24* mutation may be on the deleted part.

Cozzarelli postulates two domains in the *gyrB* product (4, 7). One domain represented by *ν* is sufficient for binding to the *gyrA* product and reconstitution of breakage reunion activity of DNA. The other domain contains the ATP binding site or allows its expression in energy-requiring reactions. It is possible that *nal-31* and *nal-24* are situated somewhere on a part of the *gyrB* gene encoding *ν*, for the mutation affects markedly the sensitivity to pyridonecarboxylic acid derivatives but slightly the sensitivity to NB, which binds the ATP binding site.

It remains to be clarified whether the *gyrB* product has an NA binding site. Each gyrase subunit is inactive alone, but reconstitutes gyrase activity with the other subunit. This fact suggests a conformational change of the subunits upon reconstitution. A mutational aberration of subunit B may affect the conformation of gyrase so that the enzyme is resistant to NA. This may be the case in subunit A. In this connection, it is informative that a *gyrB* mutant, LE701, is resistant to both NB and NA (12), and a *gyrA* mutant, KL166, is more susceptible to NB than the wild strain (5).

In *Bacillus subtilis*, gyrase from an NB resistance (*novA*) mutant or an NA resistance (*nalA*) mutant is resistant to NB or NA, and both the *novA* and *nalA* genes are very close each other in the proximity of the origin of DNA replication (49). The replication origin of chromosomal DNA, *oriC*, is mapped at about 83 min in *E. coli* K-12 (2). So, the situation of *gyrB* and *nal-31* in *E. coli* is very similar to that of *novA* and *nalA* in *B. subtilis*. There is circumstantial evidence that *B. subtilis* gyrase is involved in the initiation of DNA replication (49). The rapidity of the inhibition of DNA replication by pyridonecarboxylic acid derivatives (43) and coumermycin A_1 (40, 41) or by mutational inactivation of the *gyrA* protein (25, 38) means a role of gyrase or related enzymes in DNA replication but would not rule out their role in the process of initiation of DNA replication. In a temperature-sensitivity mutation in the *gyrB* gene, initiation of DNA replication seems to be affected at the high temperature (37). Moreover, it seems that a functional relationship exists between the product of the *dnaA* gene and DNA gyrase (13). Gyrase or related enzymes may be important for DNA replication fork movement and its initiation.

The *nal-31* and *nal-24* mutations appear to be useful for analyzing the structure of the *gyrB* gene and the role of its product on the metabolic processes involving gyrase and related enzymes.

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LITERATURE CITED

1. **Aaij, C., and P. Borst.** 1972. The gel electrophoresis of DNA. Biochim. Biophys. Acta **269**:192-200.
2. **Bachman, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116-167.
3. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83-88.
4. **Brown, P. O., C. L. Peebles, and N. R. Cozzarelli.** 1979. A topoisomerase from *Escherichia coli* related to DNA gyrase. Proc. Natl. Acad. Sci. U.S.A. **76**:6110-6114.
5. **Chao, L.** 1978. An unusual interaction between the target of nalidixic acid and novobiocin. Nature (London) **271**: 385-386.
6. **Cozzarelli, N. R.** 1977. The mechanism of action of inhibitors of DNA synthesis. Annu. Rev. Biochem. **46**: 641-668.
7. **Cozzarelli, N. R.** 1980. DNA gyrase and the supercoiling of DNA. Science **207**:953-960.
8. **Crumplin, G. C., and J. T. Smith.** 1976. Nalidixic acid and bacterial chromosome replication. Nature (London) **260**:643-645.
9. **Deitz, W. H., J. H. Bailey, and E. J. Froelich.** 1964. In vitro antibacterial properties of nalidixic acid, a new drug active against gram-negative organisms, p. 583-587. Antimicrob. Agents Chemother. 1963.
10. **Deitz, W. H., T. M. Cook, and W. A. Goss.** 1966. Mechanism of action of nalidixic acid on *Escherichia coli*. III. Conditions required for lethality. J. Bacteriol. **91**:768-773.
11. **Drlica, K., and M. Snyder.** 1978. Superhelical *Escherichia coli* DNA: relaxation by coumermycin. J. Mol. Biol. **120**:145-154.
12. **Fairweather, N. F., E. Orr, and I. B. Holland.** 1980. Inhibition of deoxyribonucleic acid gyrase: effect on nucleic acid synthesis and cell division in *Escherichia coli* K-12. J. Bacteriol. **142**:153-161.
13. **Filutowicz, M.** 1980. Requirement of DNA gyrase for the initiation of chromosome replication in *Escherichia coli* K-12. Mol. Gen. Genet. **177**:301-309.
14. **Gellert, M., L. M. Fisher, and M. H. O'Dea.** 1979. DNA gyrase: purification and catalytic properties of a fragment of gyrase B protein. Proc. Natl. Acad. Sci. U.S.A. **76**:6289-6293.
15. **Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. Tomizawa.** 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. U.S.A. **74**:4772-4776.
16. **Gellert, M., K. Mizuuchi, M. H. O'Dea, and H. A. Nash.** 1976. DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. U.S.A. **73**:3872-3876.
17. **Gellert, M., M. H. O'Dea, T. Itoh, and J. Tomizawa.** 1976. Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. U.S.A. **73**:4474-4478.
18. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064-1066.
19. **Hansen, F. G., and K. von Meyenburg.** 1979. Characterization of the *dnaA*, *gyrB* and other genes in the *dnaA* region of the *Escherichia coli* chromosome on specialized transducing phage λ*tna*. Mol. Gen. Genet. **175**:135-144.
20. **Higgins, N. P., C. L. Peebles, A. Sugino, and N. R. Cozzarelli.** 1978. Purification of the subunits of *Escherichia coli* DNA gyrase and reconstitution of enzymatic activity. Proc. Natl. Acad. Sci. U.S.A. **75**:1773-1777.
21. **Hirose, S.** 1973. Method in nucleic acid research, p. 4-8. Kyoritsu Shuppan Co., Ltd., Tokyo.
22. **Inoue, S., T. Ohue, J. Yamagishi, S. Nakamura, and M. Shimizu.** 1978. Mode of incomplete cross-resistance among pipemidic, piromidic, and nalidixic acids. Antimicrob. Agents Chemother. **14**:240-245.
23. **Itoh, I., and J. Tomizawa.** 1977. Involvement of DNA gyrase in bacteriophage T7 DNA replication. Nature (London) **270**:78-80.
24. **Kaminsky, D., and R. I. Meltzer.** 1968. Quinolone antibacterial agents. Oxolinic acid and related compounds. J. Med. Chem. **11**:160-163.
25. **Kreuzer, K. N., K. McEntee, A. P. Geballe, and N. R. Cozzarelli.** 1978. Lambda transducing phages for the *nalA* gene of *Escherichia coli* and conditional lethal *nalA* mutations. Mol. Gen. Genet. **167**:129-137.
26. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:335-341.
27. **Lesher, G. Y., E. J. Froelich, M. D. Gruett, J. H. Bailey, and R. P. Brundage.** 1962. 1,8-Naphthyridine derivatives. A new class of chemotherapeutic agents. J. Med. Pharm. Chem. **5**:1063-1065.
28. **Marians, K. J., J. Ikeda, S. Schlagman, and J. Hurwitz.** 1977. Role of DNA gyrase in φX replicative-form replication *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **74**: 1965-1968.
29. **Matsumoto, J., and S. Minami.** 1975. Pyrido[2,3-d]py-

rimidine antibacterial agents. 3. 8-Alkyl- and 8-vinyl-5,8-dihydro-5-oxo-2-(1-piperazinyl)pyrido[2,3-d]pyrimidine-6-carboxylic acids and their derivatives. J. Med. Chem. **18**:74–79.

30. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
31. **Miki, T., S. Hiraga, T. Nagata, and T. Yura.** 1978. Bacteriophage λ carrying the *Escherichia coli* chromosomal region of the replication origin. Proc. Natl. Acad. Sci. U.S.A. **75**:5099–5103.
32. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Minami, S., T. Shono, and J. Matsumoto.** 1971. Pyrido[2,3-d]pyrimidine antibacterial agents. II. Piromidic acid and related compounds. Chem. Pharm. Bull. **19**: 1426–1432.
34. **Mizuuchi, K., and H. Nash.** 1976. Restriction assay for integrative recombination of bacteriophage λ DNA *in vitro*: requirement for closed circular DNA substrate. Proc. Natl. Acad. Sci. U.S.A. **73**:3524–3528.
35. **Mizuuchi, K., M. H. O'Dea, and M. Gellert.** 1978. DNA gyrase: subunit structure and ATPase activity of the purified enzyme. Proc. Natl. Acad. Sci. U.S.A **75**:5960–5963.
36. **Murakami, A., H. Inokuchi, Y. Hirota, H. Ozeki, and H. Yamagishi.** 1980. Characterization of the *dnaA* gene carried by lambda transducing phage. Mol. Gen. Genet. **180**:235–247.
37. **Orr, E., N. F. Fairweather, I. B. Holland, and R. H. Pritchard.** 1979. Isolation and characterization of a strain carrying a conditional lethal mutation in the *cou* gene of *Escherichia coli* K-12. Mol. Gen. Genet. **177**: 103–112.
38. **Peebles, C. L., N. P. Higgins, K. N. Kreuzer, A. Morrison, P. O. Brown, A. Sugino, and N. R. Cozzarelli.** 1978. Structure and activities of *Escherichia coli* DNA gyrase. Cold Spring Harbor Symp. Quant. Biol. **43**:41–52.
39. **Randerath, K.** 1970. An evaluation of film detection methods for weak β-emitters, particularly tritium. Anal. Biochem. **34**:188–205.
40. **Ryan, M. J.** 1976. Coumermycin A_1: a preferential inhibitor of replicative DNA synthesis in *Escherichia coli*. I. *In vivo* characterization. Biochemistry **15**:3769–3777.
41. **Ryan, M. J., and R. D. Wells.** 1976. Coumermycin A_1: a preferential inhibitor of replicative DNA synthesis in *Escherichia coli*. II. *In vitro* characterization. Biochemistry **15**:3778–3782.
42. **Sakakibara, Y., and J. Tomizawa.** 1974. Replication of colicin E1 plasmid DNA in cell extracts. Proc. Natl. Acad. Sci. U.S.A. **71**:802–806.
43. **Sammes, P. G.** 1980. Topics in antibiotic chemistry, vol. 3, p. 13–38. John Wiley & Sons, Inc., New York.
44. **Sanzey, B.** 1979. Modulation of gene expression by drugs affecting deoxyribonucleic acid gyrase. J. Bacteriol. **138**:40–47.
45. **Shimizu, M., S. Nakamura, and Y. Takase.** 1971. Piromidic acid, a new antibacterial agent: antibacterial properties, p. 117–122. Antimicrob. Agents Chemother. 1970.
46. **Shimizu, M., Y. Takase, S. Nakamura, H. Katae, A. Minami, K. Nakata, S. Inoue, M. Ishiyama, and Y. Kubo.** 1975. Pipemidic acid, a new antibacterial agent active against *Pseudomonas aeruginosa*: in vitro properties. Antimicrob. Agents Chemother. **8**:132–138.
47. **Smith, C. L., M. Kubo, and F. Imamoto.** 1978. Promoter-specific inhibition of transcription by antibiotics which act on DNA gyrase. Nature (London) **275**:420–423.
48. **Snyder, M., and K. Drlica.** 1979. DNA gyrase on the bacterial chromosome: DNA cleavage induced by oxolinic acid. J. Mol. Biol. **131**:287–302.
49. **Sugino, A., and K. F. Bott.** 1980. *Bacillus subtilis* deoxyribonucleic acid gyrase. J. Bacteriol. **141**:1331–1339.
50. **Sugino, A., C. L. Peebles, K. N. Kreuzer, and N. R. Cozzarelli.** 1977. Mechanism of action of nalidixic acid: purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc. Natl. Acad. Sci. U.S.A. **74**:4767–4771.
51. **Tomizawa, J.** 1976. Experiments in bacteriophages, p. 101–108. Iwanami Shoten, Tokyo.
52. **Yang, H. L., K. Heller, M. Gellert, and G. Zubay.** 1979. Differential sensitivity of gene expression *in vitro* to inhibitors of DNA gyrase. Proc. Natl. Acad. Sci. U.S.A. **76**:3304–3308.
53. **Young, E. T., II, and R. L. Sinsheimer.** 1964. Novel intracellular forms of lambda DNA. J. Mol. Biol. **10**: 562–564.