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Nalidixic acid-resistant mutations of the *gyrB* gene of *Escherichia coli*

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Summary. DNA fragments of 3.4 kb containing the *gyrB* gene were cloned from *Escherichia coli* KL-16 and from spontaneous nalidixic acid-resistant mutants. The mutations (*nal-24* and *nal-31*) had been determined to be in the *gyrB* gene by transduction analysis. Nucleotide sequence analysis of the cloned DNA fragments revealed that *nal-24* was a G to A transition at the first base of the 426th codon of the *gyrB* gene, resulting in an amino acid change from aspartic acid to asparagine, and *nal-31* was an A to G transition at the first base of the 447th codon, resulting in an amino acid change from lysine to glutamic acid. This indicates that mutations in the *gyrB* gene are responsible for nalidixic acid resistance.

Key words: Nalidixic acid resistance – *gyrB* gene – Nucleotide sequence – *Escherichia coli*

Introduction

Pyridonecarboxylic acids (PCA) or quinolones are a group of antibacterial agents whose molecular target is considered to be DNA gyrase (Gellert et al. 1977; Sugino et al. 1977). The DNA gyrase of *Escherichia coli* consists of two subunits, A and B (Higgins et al. 1978; Mizuuchi et al. 1978; Sugino et al. 1977), which are the products of the *gyrA* and *gyrB* genes located at 48 and 83 min, respectively (Bachmann 1983). Nalidixic acid (NA)-resistance is generally considered to be conferred by a *gyrA* (formerly *nalA*) mutation (Gellert et al. 1977) and novobiocin (NB)-resistance by a *gyrB* (formerly *cou*) mutation (Gellert et al. 1976).

Newer compounds of this group, pipemidic acid (PPA), norfloxacin, enoxacin, ofloxacin, ciprofloxacin etc. are incompletely cross-resistant with NA (Shimizu et al. 1975; Nakamura et al. 1983; Smith 1984). During a study on the mode of the incomplete cross-resistance, new NA-resistant mutations, *nal-24* and *nal-31* were found at approximately 83 min (formerly 82 min) (Inoue et al. 1978). The *nal-24* mutation confers resistance to both NA and PPA, and the *nal-31* mutation resistance to NA and at the same time hypersensitivity to PPA (Inoue et al. 1978). The DNA gyrase activities of the mutants are resistant to NA and resistant to or hypersensitive to PPA (Yamagishi et al. 1981). However, these mutations do not cause NB resistance.

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P1 transduction showed that the mutations were co-transducible with *dnaA* (83 min) and transduction with transducing λ phages carrying the partially deleted *gyrB* gene suggested that the *nal-31* mutation was probably on the *gyrB* gene (Yamagishi et al. 1981). To clarify the sites and types of the *nal* mutations, we cloned the *gyrB* genes from the wild-type strain and the *nal-24* and *nal-31* mutants.

Materials and methods

Bacterial strains and plasmids. All the bacterial strains used were *E. coli* K12 derivatives of which the relevant genotypes and derivations are listed in Table 1. The *recA*$^-$ strains JC1552-O3, JC1552-C3, JC1552-D3 and LC257-N3 were isolated as follows: strain JC1552 was treated with trimethoprim and a *Thy*$^-$ strain, JC1552-O2, was selected. The *nal-24* and *nal-31* derivatives of strain JC1552-O2, JC1552-D2 and JC1552-C2, were made by P1 transduction of the *nal* mutations from N-24 and N-31 by the method of Miller (1972). LC257-N was selected from LC257 on LB-agar containing 800 μg of NB per ml and then a streptomycin (SM)-resistant mutant of LC257-N, LC257-N1, was selected on LB-agar containing 100 μg of SM per ml. These *Thy*$^-$ and SM-resistant strains, JC1552-O2, JC1552-D2, JC1552-C2 and LC257-N1 were crossed with the *Thy*$^+$ and SM-sensitive strain AR-10, and for each a *Thy*$^+$, SM-resistant and UV-sensitive transconjugant was selected and designated as JC1552-O3, JC1552-D3, JC1552-C3 and LC257-N3, respectively. Plasmid vector pBR322 was purchased from Bethesda Research Laboratories, Inc.

Chemicals and enzymes. NA (Lesher et al. 1962) was synthesized in our laboratories. Sodium ampicillin was purchased from Meiji Seika Kaisha, Ltd., sodium novobiocin, lysozyme (grade I), and RNase A (typeI–A) from Sigma Chemical Co. and the other reagents (guaranteed reagent) from Nakarai Chemicals, Ltd. Restriction endonucleases, T4 DNA ligase and Bal-31 nuclease and the sequencing kit were obtained from Takara Shuzo Co., Ltd. and α^{32}P-dCTP (>400 Ci/mmol) from Amersham International.

Media. LB-medium or -agar and minimal medium were prepared as described previously (Inoue et al. 1978). Supplements were added, if necessary, at the following concentrations: 50 μg/ml ampicillin; 20 μg/ml amino acids; 1 μg/ml thiamine hydrochloride; 50 μg/ml thymine.

Table 1. Bacterial strains used

Strain	Relevant genotype	Derivation
AR-10(KL-16-99)	HfrH *recA*	Low (1968)
JC1552	F^- *rpsL*	Bachmann (1972)
JC1552-O2	Thy^- derivative of JC1552	This study
JC1552-O3	Thy^+ *recA* derivative of JC1552-O2	This study
JC1552-C2	*nal-31* derivative of JC1552-O2	This study
JC1552-C3	Thy^+ *recA* derivative of JC1552-C2	This study
JC1552-D2	*nal-24* derivative of JC1552-O2	This study
JC1552-D3	Thy^+ *recA* derivative of JC1552-D2	This study
JC3913	F^- *recF uvrA*	Kato et al. (1977)
KL-16	HfrH	Low (1968)
LC257	F^- *dnaA46 thy*	From H. Ryo
LC257-N	*gyrB-15* derivative of LC257	This study
LC257-N1	*rpsL* derivative of LC257-N	This study
LC257-N3	Thy^+ *recA* derivative of LC257-N1	This study
N-24	*nal-24* derivative of KL-16	Inoue et al. (1978)
N-31	*nal-31* derivative of KL-16	Inoue et al. (1978)

Preparation of plasmid and chromosomal DNA. Plasmid DNA was prepared by a rapid boiling method as described by Holmes and Quigley (1981), or by the method of Wilkie et al. (1979). Chromosomal DNA was prepared by the method of Cosloy and Oishi (1973). Electroelution of DNA was carried out essentially as described by Maniatis et al. (1982).

DNA sequencing. DNA sequencing was carried out by the chain-termination method with M13 phage vectors (Messing 1983; Sanger et al. 1977).

Phenotype check. The $RecF^+$ phenotype was tested by UV sensitivity as described by Murakami et al. (1980). The $GyrB^+$ phenotype was tested by NB sensitivity. NB- or NA-sensitivity was defined by inability to grow on LB-agar containing 200 μg/ml NB or 25 μg/ml NA.

Results

Cloning of the gyrB genes

It is known that the *dnaA, dnaN, recF* and *gyrB* genes are clustered on the 13 kb *Hin*dIII fragment of the *E. coli* K-12 chromosome (Hansen and Meyenburg 1979; Kimura et al. 1979; Miki et al. 1979; Murakami et al. 1980; Schaus et al. 1981; Hansen et al. 1982; Blanar et al. 1984; Mizuuchi et al. 1984; Ohmori et al. 1984). Therefore, chromosomal DNA of *E. coli* KL-16 having the wild-type *dnaA* and *gyrB* genes was digested with *Hin*dIII and the digested DNA fragments were inserted into the *Hin*dIII site of pBR322. *E. coli* LC257-N3, a *dnaA* and *gyrB* double mutant which is unable to grow at 42° C and is resistant to NB, was transformed with the resultant recombinant plasmids, and ampicillin-resistant (pBR322 marker) and temperature-resistant ($DnaA^+$ phenotype) colonies were selected. The selected colonies were checked for NB sensitivity ($GyrB^+$ phenotype). Plasmids were isolated from the selected NB-sensitive strains because the partially diploid strains with both the wild-type and NB-resistant *gyrB* genes are known to be NB-sensitive phenotypically (Hansen and Meyenburg 1979). JC3913, a UV-sensitive *recF* mutant (Kato et al. 1977), was transformed with the isolated plasmids and transformants were checked for UV resistance, ($RecF^+$ phenotype). One of the transformants showing UV resistance was selected and its plasmid was designated pJM2-9. This plasmid possessed $dnaA^+$, $recF^+$ and $gyrB^+$ transducing activities indicating that it was carrying a wild-type chromosomal DNA fragment containing the *dnaA-gyrB* region. The restriction map of the 13 kb DNA insert of pJM2-9 is shown in Fig. 1.

The corresponding DNA fragments containing the *dnaA-gyrB* region were also cloned from the *nal-24* mutant, N-24, and the *nal-31* mutant, N-31. The plasmids carrying the 13 kb DNA fragments with the *nal-24* and *nal-31* mutations were designated as pJD2-9 and pJC2-9, respectively. Transformants harbouring one of the plasmids generally showed poor growth compared with their original host strains.

Location of the nal mutations

To locate the *gyrB* gene, pJM2-9 was partially digested with *Ava*I and/or *Eco*RI followed by self-ligation. The resultant plasmids with shorter DNA fragments were designated as pJA21, pJA83, pJE5 and pJE3. Each plasmid was introduced into LC257-N3 and the transformant was checked for NB sensitivity. As shown in Fig. 1, pJA21 with a deletion of the $AvaI_3$–$AvaI_4$ fragment conferred NB sensitivity while pJA83 lacking the $AvaI_2$–$AvaI_4$ fragment showed no such activity. Plasmid pJE5 lacking the $EcoRI_1$–$EcoRI_2$ fragment present in pJA21 showed the same activity but pJE3 lacking the $EcoRI_1$–$EcoRI_3$ fragment did not. Plasmid pJE5, was cleaved with *Xho*I, digested appropriately with Bal-31 and self-ligated. The resultant plasmid pJB11 conferred NB sensitivity. The result shows that the wild-type *gyrB* gene is located on the 3.4 kb chromosomal fragment carried by pJB11. This plasmid also conferred NA sensitivity when introduced into the *nal-24* mutant, JC1552-D3, and the *nal-31* mutant, JC1552-C3.

Similar plasmids, pJD11-2 and pJC11-6, were constructed by replacing a 2.8 kb *Sma*I–$AvaI_3$ fragment of pJB11 by the corresponding fragments of pJD2-9 and pJC2-9, respectively. The plasmids, pJD11-2 and pJC11-6, conferred NB sensitivity but not NA sensitivity when introduced into hosts having the same or heterogeneous *nal* mu-

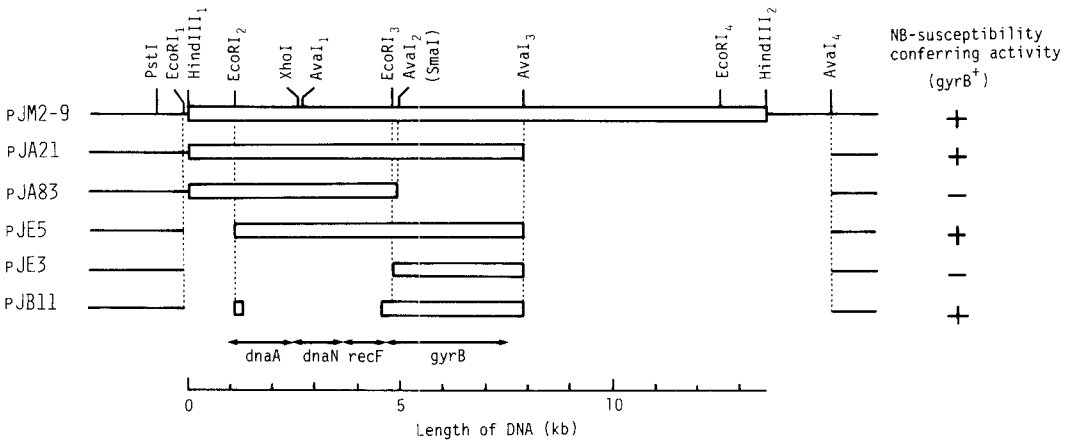

Fig. 1. Restriction maps of the genes carried by plasmid pJM2-9 and its derivatives. *Blocks* and *lines* represent DNAs from the *Escherichia coli* chromosome and the vector pBR322, respectively. Deletions are shown as *gaps*. The symbols + or − indicate that the plasmid does or does not possess the ability to confer NB sensitivity, respectively

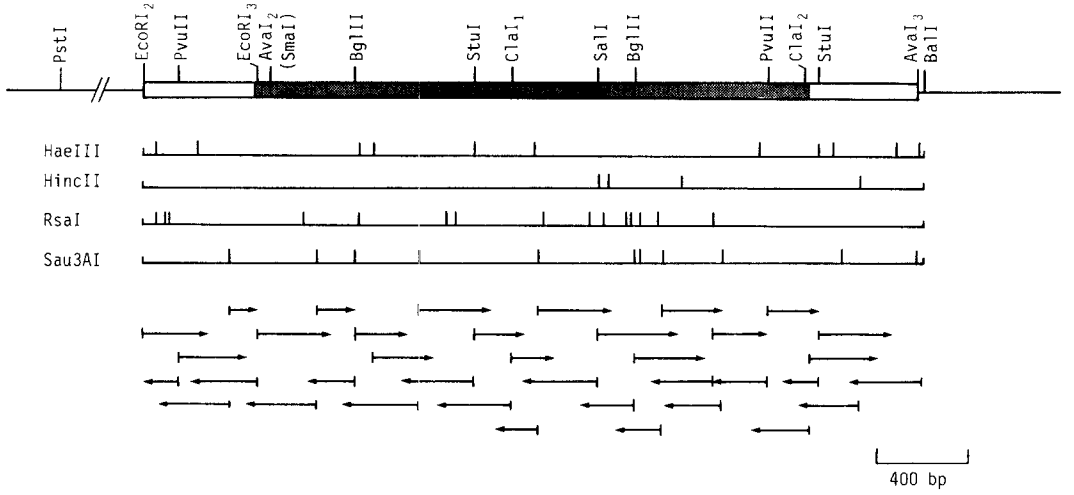

Fig. 2. Restriction map of the 3.4 kb fragment from pJB11 and the strategy for determining the nucleotide sequence. The *shadowed box* indicates the *gyrB* structural gene. Cleavage sites for restriction enzymes are shown as *vertical lines*. The *arrows* below the map indicate the direction and extent of sequence analysis

tations. As the wild-type *gyrB* gene is dominant to the *nal-24* and *nal-31* mutant genes, the location of the *nal* mutations was determined as follows. The *Pst*I–*Cla*I$_1$ (see Fig. 2) fragment of pJB11 was replaced by the corresponding fragments of pJD11-2 and pJC11-6 and the resultant plasmids were checked for ability to confer NA sensitivity. Both of them did so demonstrating that the *Pst*I–*Cla*I$_1$ fragments of pJD11-2 and pJC11-6 do not contain the *nal* mutations.

Next, the *Sal*I–*Ava*I$_3$ (see Fig. 2) fragment of pJB11 was replaced by the corresponding fragments of pJD11-2 and pJC11-6. The same result was obtained in this case also. From these results, it was concluded that the *nal-24* and *nal-31* mutations were probably located on the 0.4 kb *Cla*I$_1$–*Sal*I fragment of the *gyrB* gene.

Nucleotide sequence of the gyrB genes

The nucleotide sequence of the wild-type *gyrB* gene and its pJB11 flanking regions were determined by the strategy shown in Fig. 2. As shown in Fig. 3, the nucleotide sequence contains only one open reading frame long enough to encode a polypeptide of 804 amino acids. The molecular weight from the deduced amino acid sequence is 89,969. This value is in good agreement with the value of 91,000 daltons for the polypeptide detected by maxicell analysis (data not shown). A promoter sequence (Pribnow box, TAAAAT and −35 region; TTCGAA) is located in the *recF* structured structural gene. A long inverted repeated sequence which might serve as a transcription terminator is seen at nucleotides 2547–2576 (underlined part in Fig. 3).

The nucleotide sequences of the 0.4 kb *Cla*I$_1$–*Sal*I fragments from pJD11-2 and pJC11-6 were determined by the same sequencing strategy and compared with that of the corresponding fragment from pJB11. As shown in Fig. 3, a single G to A transition was detected at nucleotide 1276 in the fragment with the *nal-24* mutation. This caused an amino acid change from aspartic acid to asparagine. A single A to G transition at nucleotide 1339 in the fragment with the *nal-31* mutation caused an amino acid change from lysine to glutamic acid. To check the presence of mutations at other sites, the nucleotide sequences of the 2.8 kb *Sma*I–*Ava*I$_3$ fragments of pJD2-9 and pJC2-9 were determined. Each of the sequences was exactly the same as that of pJB11 except for the above-mentioned mutation sites demonstrating that the *nal* mutations were conferred by single point mutations in the middle part of the *gyrB* gene.

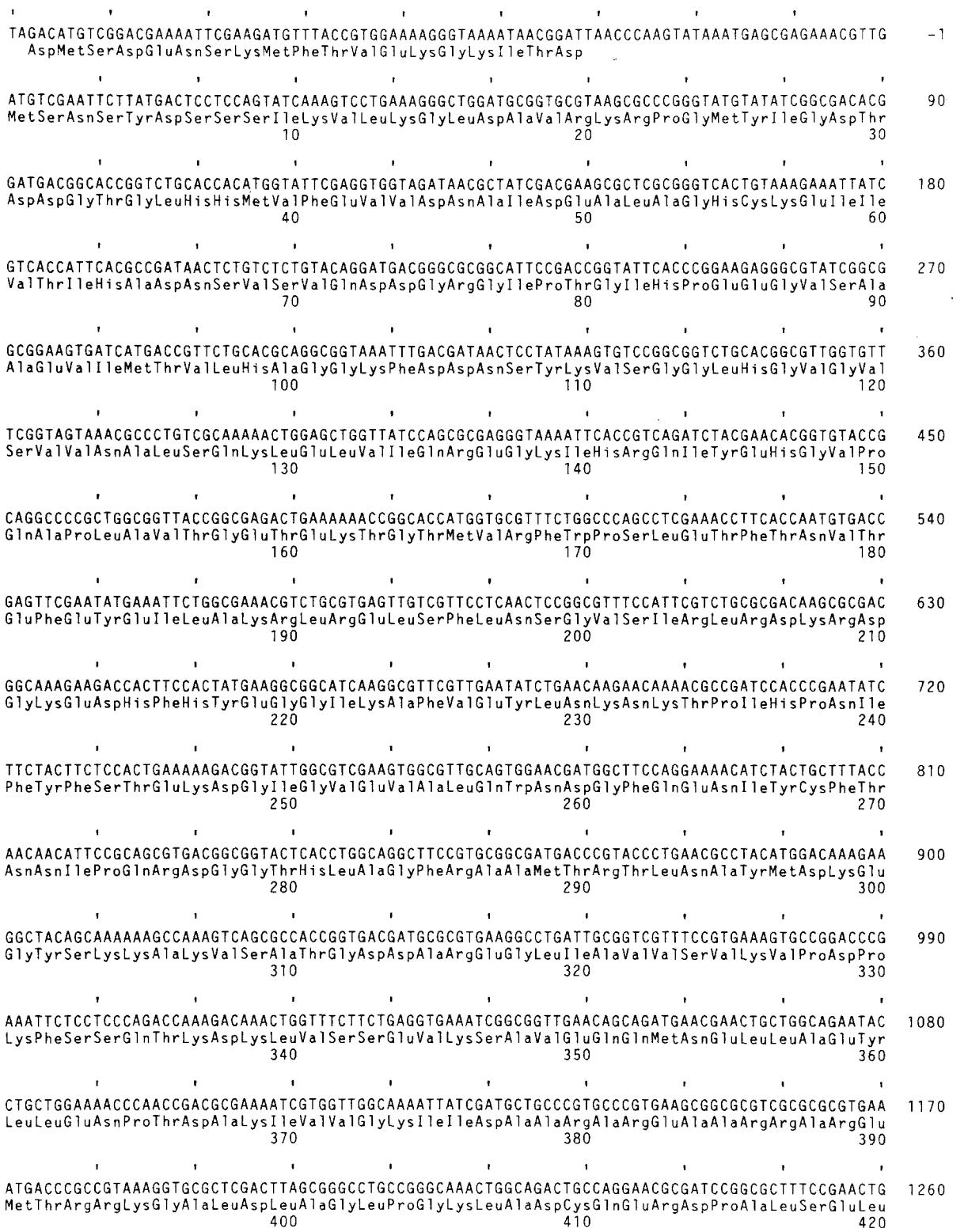

Fig. 3. Nucleotide sequence of the *gyrB* gene. The nucleotide sequence of the sense strand of the *gyrB* gene is presented from the 5′ (left) to 3′ (right) end. The deduced amino acid sequence of the *gyrB* gene product is given below the DNA sequence. Nucleotide positions are numbered, starting at the first A of the ATG initiation codon. The positions of the two mutation sites of *nal-24* and *nal-31* and the changed nucleotides and amino acids are also indicated

Discussion

It is well known that DNA gyrase is inhibited by the PCA and NB groups of antibacterial agents. Early studies using NA- and NB-resistant mutants indicated that NA resistance was conferred by *gyrA* mutations and NB resistance by *gyrB* mutations (Gellert et al. 1976; Gellert et al. 1977). However, it was found later that some NA-resistant mutations (*nal-24* and *nal-31*) mapped near the *gyrB* gene and their DNA gyrase activities were resistant to NA but not

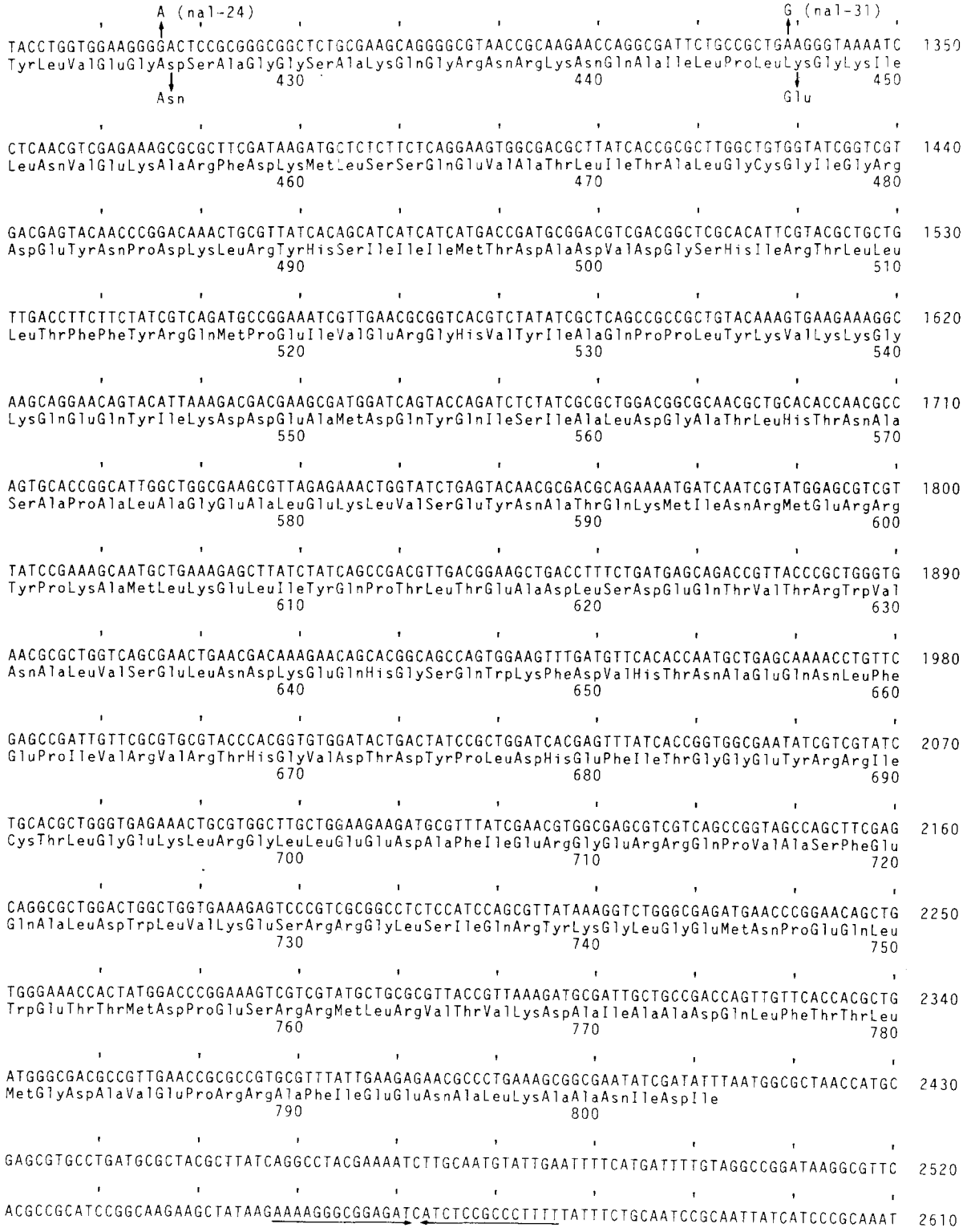

Fig. 3

resistant to NB in vivo (Yamagishi et al. 1981). The result strongly suggested that the NA resistance might be conferred not only by a *gyrA* mutation but also by a *gyrB* mutation. However, there was a slight possibility that the *nal* mutations were on some other gene near *gyrB* whose mutations affected the sensitivity of DNA gyrase to PCA in vivo, because neither the *gyrB* gene nor the *gyrB* gene product had yet been isolated from the mutants.

In order to eliminate this possibility, we cloned the *gyrB* gene from the wild-type strain and the *nal* mutants. The nucleotide sequence shown in Fig. 3 contains a sequence identical to the upstream region of the *gyrB* gene reported by Adachi et al. (1984) except for differences at two sites: our sequences at nucleotides 151–152 and 167–168 are GC and GT while theirs are CG and TG. Such identity provided us with confidence that the cloned sequence was the whole

Fig. 4. Comparison of the amino acid sequence of the *Escherichia coli gyrB* gene (upper) with the *Bacillus subtilis gyrB* gene (lower) (Moriya et al. 1985). The sequences have been aligned to give maximal homology by introducing a gap into the *B. subtilis gyrB* gene. Identical amino acids are represented by the symbol *

gyrB gene of which the primary structure was elucidated for the first time in this paper. The calculated molecular weight (about 90,000) of the *gyrB* gene product is lower than that (about 95,000) reported by Gellert (1981) but within experimental variation.

Recently, the *gyrB* gene of *Bacillus subtilis* has been cloned and sequenced (Lampe and Bott 1985; Moriya et al. 1985; Ogasawara et al. 1985). The deduced *gyrB* gene product consists of 638 amino acids with a molecular weight of about 72,000. There is about 60% homology between the deduced amino acid sequences of the *E. coli* and *B. subtilis gyrB* genes, provided that a sequence corresponding to 169 amino acids (from amino acid 564 to 732) in the middle part of the *E. coli gyrB* gene is excluded (Fig. 4). The conserved sequences may be functionally important and the unconserved sequence may be dispensable. This assumption is supported by the fact that purified *E. coli* subunit A and purified *B. subtilis* subunit B reconstitute active DNA gyrase (Orr and Staudenbauer 1982).

Figure 3 also shows that *nal-24* is a G to A transition at nucleotide 1276 of the *gyrB* gene, resulting in an amino acid change from aspartic acid to asparagine at amino acid 426, and *nal-31* is an A to G transition at nucleotide 1339, resulting in an amino acid change from lysine to glutamic acid at amino acid 447.

One interesting fact is that the sites of the *nal-24* and *nal-31* mutations are close to each other despite a difference in their resistance pattern and the mutation sites are in the conserved sequence (from amino acid 1 to 563). Cozzarelli (1980) postulates two domains in the *gyrB* gene product. One domain, represented by *v*, is sufficient for binding to the *gyrA* product and the reconstitution of the breakage-reunion activity of DNA. The other domain contains the ATP binding site and allows its expression in energy-requiring reactions. We have previously pointed out that the *nal-24* and *nal-31* mutations are situated somewhere on a part of the *gyrB* gene encoding *v*, for the mutations markedly affect the sensitivity to PCA but only slightly that to NB (which binds the ATP binding site). It is likely that the above conserved sequence (from amino acid 1 to 563) encodes *v* and the other (from amino acid 733 to 804) encodes the ATP binding site, though further studies are required to confirm this.

Also interesting is the fact that both mutations would cause changes in electric charge. The *nal-24* mutation which causes a decrease in minus charge confers resistance to both NA and PPA. The *nal-31* mutation which causes an increase in minus charge confers resistance to NA but hypersensitivity to PPA. The *nal-31* mutation also confers resistance to oxolinic acid, piromidic acid, cinoxacin, flumequine and rosoxacin, and hypersensitivity to norfloxacin, enoxacin, ofloxacin and ciprofloxacin (Inoue et al. 1982; Smith 1984). All of the compounds to which the *nal-31* mutation confers resistance have only a carboxyl group in their molecules which could be negatively charged while all the compounds to which the *nal-31* mutation confers hypersensitivity have both a carboxyl group and a piperazinyl group which could be positively charged. These facts suggest that electric charge may be in some way related to drug-gyrase interaction. Apart from the biochemical mechanism of PCA resistance, the present study clearly indicates that *gyrB* mutations are responsible for resistance to PCA.

This result combined with the evidence accumulated up to now indicates that DNA gyrase could become resistant to PCA by a mutation at either the *gyrA* or *gyrB* gene.

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