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Bacillus subtilis Deoxyribonucleic Acid Gyrase

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Bacillus subtilis 168 was shown to contain a deoxyribonucleic acid (DNA) gyrase activity which closely resembled those of the enzymes isolated from *Escherichia coli* and *Micrococcus luteus* in its enzymatic requirements, substrate specificity, and sensitivity to several antibiotics. The enzyme was purified from the wild type and nalidixic acid-resistant and novobiocin-resistant mutants of *B. subtilis* and was functionally characterized in vitro. The genetic loci *nalA* and *novA* but not *novB* were shown to code for portions of the functional gyrase. Enzyme from the antibiotic-resistant mutants was resistant to the drug in vitro. The most striking observation was the remarkable similarity between the *B. subtilis* enzyme and other DNA gyrases, especially with respect to the oxolinic acid-induced DNA cleavage in the presence of sodium dodecyl sulfate. All of the enzymes appeared to possess the same specificity of cutting sites regardless of the source or type of DNA used. This result implies that gyrase binding to DNA is highly specific.

The enzyme DNA gyrase is a topoisomerase (30) since it directly affects the extent of DNA supercoiling. Although first characterized because it catalyzed the supercoiling of double-stranded DNA in the presence of ATP (6), more recent studies have been shown that gyrase has at least four additional activities. In *Escherichia coli* these include: relaxation of supercoiled DNA in the absence of ATP; double-strand cleavage of DNA induced by nalidixic acid (Nal) or oxolinic acid in the presence of sodium dodecyl sulfate; a DNA-dependent ATPase activity; and site-specific binding to DNA (22). *E. coli* DNA gyrase is an essential enzyme (16) with important roles in DNA replication, recombination, repair, and transcription (3a).

E. coli DNA gyrase has been shown to be encoded by two distinct genes. One subunit of the DNA gyrase molecule is a 105,000-dalton product of the *nalA* gene, a protein which determines resistance to nalidixic acid and to oxolinic acid (5, 14, 27). The locus which determines resistance to coumerycin A and to novobiocin (Nov) controls synthesis of the other subunit, a protein of approximately 95,000 daltons (7, 14, 20). In vitro the active form of DNA gyrase is probably a tetramer consisting of two subunit proteins of each type (22). Genetic studies have shown that the loci which encode the information for these two proteins are well separated on the genetic map and separately controlled (22). Furthermore, recombination analysis has demonstrated that at least for nalidixic acid (and probably for novobiocin as well) the wild-type (sensitive) allele is dominant, a feature which explains the prolonged lag time for expression of the recombinant phenotype and the disproportionately low recovery of Nal^r recombinants (11, 22). Although other mutations have been described which result in low-level resistance to these antibiotics, they are believed to affect genes controlling permeability, surface structure, etc. DNA gyrase from *Micrococcus luteus* has been purified and shown to be sensitive to both novobiocin and nalidixic acid. However, no genetic characterization has been done for this system (17).

DNA gyrase from *Bacillus subtilis* has not previously been characterized. Although mutants conferring resistance to novobiocin or nalidixic acid have been described, no biochemical lesion has been associated with those mutations (1a, 13). Aside from the intrinsic biochemical comparisons of enzyme similarities between gram-positive and gram-negative bacteria, there are several reasons for initiating an intensive characterization of *B. subtilis* DNA gyrase. Novobiocin has been shown to permit replication of only a limited segment of the *B. subtilis* chromosome immediately adjacent to the site where chromosome replication is initiated (21). *B. subtilis* is physiologically more complex than *E. coli*, since it initiates a program of differential gene expression leading to the synthesis of endospores when nutrient limitation causes cellular growth and DNA synthesis to cease (23). Nalidixic acid and novobiocin are known to act differentially on germinating spores, on vegeta-

tive cells, and on the initiation of sporulation (8, 9); hence, studies of DNA gyrase as it influences the state of the chromosome are justified in attempts to explain the events important in regulating the differentiated state.

Two Nov^r loci have been reported for *B. subtilis*. One is closely linked to the single locus for Nal^r, suggesting that the expression of these two genes is coordinately controlled (31). These two loci are located very close to the origin of DNA replication (14). This region has been extremely difficult to characterize by classic genetic studies, perhaps due to its proximity to the origin or to the complexity of function and the essentiality of DNA gyrase (21).

MATERIALS AND METHODS

Bacteria. *B. subtilis* 168 was originally obtained from J. Spizizen. The Nal^r strain, N-10, was obtained by plating the N. Sueoka strain, MU8U5U1 (*leu ilv met*) (13), directly onto plates of tryptose-blood agar base (Difco) containing 50 μg of nalidixic acid per ml. Strains B4 (*gua trp*) and JH855 (*gua spo purA*) were obtained from J. Hoch. The isogenic series of "kit strains 1 through 9," which collectively encircles the *B. subtilis* genome for transduction purposes (4), was obtained from R. Dedonder. The *novA trpC2* strain, referred to here as Nov^r-S, was obtained from N. Sueoka, and a similar strain, designated Nov^r-O, was obtained from C. Orrego. Transduction crosses with phage PBS1 were performed as described by Hoch et al. (15).

Media. The minimal medium of Spizizen (24) was supplemented with glucose and amino acids or bases. Tryptose-blood agar base was used as a rich medium in plates, and Penassay broth (antibiotic assay broth no. 3, Difco) was used for liquid cultures. For Nov^r and Nal^r strains, 5.0 μg of novobiocin per ml and 50 μg of nalidixic acid per ml, respectively, were added to antibiotic assay broth no. 3 or tryptose-blood agar base. Large-scale cultures for enzyme purification were grown in 5.5 liters of SLBH medium (12), using a Labline SMS high-density fermentor. Cells were harvested at an optical density at 500 nm of 10 to 15; exponential growth ceased at an optical density of 500 nm of 15, yielding approximately 100 to 200 g (wet weight) of cells. No mid-course pH adjustments were made.

Chemicals. Agarose (type II), spermidine hydrochloride, nalidixic acid, were from Sigma Chemical Co. Hydroxylapatite (Bio-Gel HTP) was from Bio-Rad Laboratories. DEAE-Sephacell was obtained from Pharmacia Fine Chemicals. Oxolinic acid was a gift of Warner-Lambert Research Institute. φX174 RF1, native supercoiled ColEl DNA, and relaxed ColE1 DNA were the same as used before (26, 27). pCS540 DNA (2) pBR322, and pMB9 DNAs were prepared as described previously (25). Simian virus 40 and adenovirus type 2 DNAs and yeast tRNA were from the Bethesda Research Laboratories.

Enzyme assays. The DNA gyrase assay measures the conversion of relaxed ColE1 DNA to the supercoiled form as monitored by agarose gel electrophoresis (6). The reaction mixture (17 μl) contained 35 mM Tris-hydrochloride (pH 7.6), 6 mM $MgCl_2$, 18 mM potassium phosphate, 5 mM spermidine hydrochloride, 1.4 mM ATP, yeast tRNA (90 μg/ml), 0.2 μg of relaxed DNA, and enzyme. After the mixture incubated for 60 min at 30°C, 5 μl of a 25% (vol/vol) glycerol solution containing bromophenol blue (0.25 mg/ml) and 5% sodium dodecyl sulfate was added. The mixture was applied to a slab gel (13 by 15 by 0.4 cm) of 1.0% agarose and then subjected to electrophoresis at 40 V for 14 to 16 h at 23°C. The gels were stained with ethidium bromide and photographed by using shortwave UV illumination (6). Negatives were traced with a Joyce-Loebl microdensitometer. Under standard conditions for exposure and development, the film responded linearly to 0.3 μg of DNA. One unit of DNA gyrase converts 0.1 μg of relaxed DNA to the supercoiled form in 30 min at 30°C. The assay for breakage-rejoining was the same as for DNA gyrase, except that native ColE1 or pBR322 DNA was the substrate and ATP was omitted. Site-specific DNA breakage by DNA gyrases was carried out in the supercoiling reaction mixture, except that freshly dissolved oxolinic acid (50 μg/ml) was added, and 0.2 μg of *Eco*RI-treated DNA was used (26). The ATP-dependent incorporation of radioactive deoxyribonucleoside 5'-triphosphate into DNA in the presence and absence of novobiocin or nalidixic acid was measured by using toluenized cell suspensions (19). *B. subtilis* was grown in 40 ml of L-broth to an optical density at 650 nm of 0.7, collected by centrifugation at 5,000 rpm for 5 min in a Sorvall SS-34 rotor, and suspended in 1 ml of 0.1 M KPO_4 buffer (pH 7.4); toluene was added to 1% as described previously (19). The reaction mixture contained 80 mM KPO_4 (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM rATP, 50 μM each dCTP, dGTP, and dATP, 50 μM [^{3}H]dTTP (specific activity, 160 μCi/μmol), and 3×10^9 toluenized cells per ml. After incubation of the mixture at 30°C, acid-insoluble radioactivity was measured in a 50-μl sample.

Enzymes. Proteinase K was obtained from EM Laboratories. *E. coli* DNA gyrase purified through the hydroxylapatite step (27) was used. *Eco*RI was from H. W. Boyer, and *Hin*dIII was from the Bethesda Research Laboratories.

Partial purification of DNA gyrase from *B. subtilis*. *B. subtilis* cells grown in SLBH medium were collected by centrifugation at 8,000 rpm in a Sorvall GSA rotor, washed once with 50 mM Tris-hydrochloride (pH 7.5)–10% sucrose–1 mM EDTA, suspended in an equal weight of the same buffer, quickly frozen in a Dry Ice-ethanol bath, and stored at −80°C until use. The frozen cells (usually 100 g) were thawed at 4°C; 150 mM KCl, 5 mM dithiothreitol, and 1 mg of lysozyme per ml were successively added; and the cell suspension was incubated at 30°C for 15 min. Brij 58 (0.5%) was then added, and after 15 min the lysate was chilled to 0°C. The lysate then was subjected to centrifugation at 45,000 rpm for 50 min in a Spinco type 50 rotor (2°C). A 10% solution of neutralized Polymin P was added dropwise to the supernatant (fraction I, 100 ml) at 0°C to a final concentration of 0.5% (A. Sugino and N. R. Cozzarelli, J. Biol. Chem., in press). The crude extract was clarified by

centrifugation at 12,000 rpm for 20 min in a Sorvall GSA rotor, and the supernatant (fraction II, 100 ml) was saved. Saturated ammonium sulfate solution (pH 7.0) was added to the supernatant to 75% saturation. The precipitate was collected by centrifugation at 12,000 rpm for 20 min in a Sorvall GSA rotor, suspended in 50 ml Tris-hydrochloride (pH 7.5)-10 mM 2-mercaptoethanol-1 mM EDTA-10% glycerol (buffer A), and dialyzed against the same buffer for 4 to 6 h at 0°C (fraction III, 90 ml).

The dialysate was applied to a column (6 by 12 cm) of DEAE-Sephacell equilibrated with buffer A. The column was washed with 500 ml of buffer A containing 25 mM NaCl, and the activity was eluted with 2 liters of a 0.025 to 0.5 M NaCl linear gradient containing buffer A. DNA gyrase activity eluted at 0.25 to 0.3 M NaCl (Fig. 1A) and was concentrated by the addition of $(NH_4)_2SO_4$ to 75% saturation. The precipitate was suspended in 20 ml of a 30 mM KPO_4 buffer (pH 6.8) gradient containing 10% glycerol and 10 mM 2-mercaptoethanol. DNA gyrase activity was eluted at 0.15 to 0.25 M KPO_4 (Fig. 1B). Active fractions were pooled, concentrated by dialysis against 30% (wt/vol) of polyethyleneglycol 2000 containing buffer A, dialyzed against 50% glycerol-0.05 M Tris-hydrochloride (pH 7.5)-1 mM EDTA-10 mM 2-mercaptoethanol, and stored at −20°C (fraction V). Unless otherwise indicated, fraction V (specific activity 7,500 U/mg of protein) was used. The breakage-rejoining activity in fraction V had a specific activity of 350 U/mg of protein. Difficulty in accurately estimating the activity of DNA gyrase in crude extract prevented the inclusion of a purification table.

RESULTS

Genetic mapping of the *nal* and *nov* mutations. In *B. subtilis*, *novA* mutations confer high-level resistance to novobiocin, whereas *novB* mutations confer low-level resistance (31). To determine the gene for the target protein, as opposed to permeability mutations, the sensitivity of DNA synthesis in vitro to novobiocin was measured after the cellular permeability barriers had been breached by toluene (Table 1). Under the condition described, DNA synthesis in the presence of ATP is known to mimic the response in vivo (19). Since novobiocin and nalidixic acid or oxolinic acid inhibit DNA replication, we infer

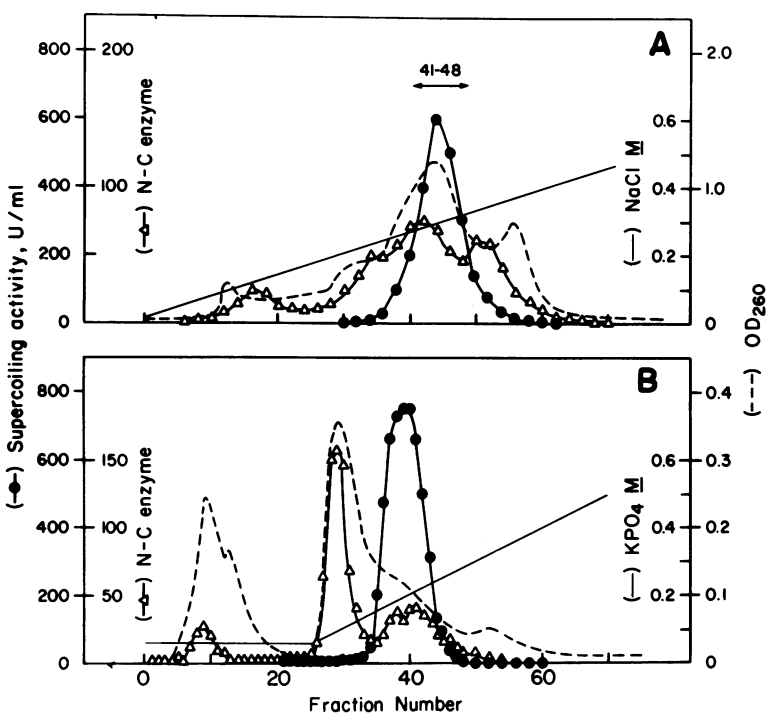

FIG. 1. *Purification of DNA gyrase from B. subtilis 168. (A) Protein extracted from B. subtilis 168 was concentrated by precipitation with $(NH_4)_2SO_4$, dialyzed, applied to a column (6 by 12 cm) of DEAE-Sephacell, and eluted with a linear NaCl gradient in 50 mM Tris-hydrochloride (pH 7.5)-10 mM 2-mercaptoethanol-1 mM EDTA-10% glycerol. The 15-ml fractions were assayed for DNA supercoiling* (○) *and relaxation of negatively supercoiled ColE1 DNA* (△). *(B) Fractions 41 through 48 in (A) were pooled, concentrated with $(NH_4)_2SO_4$, dialyzed, and applied to a column (2 by 15) cm of hydroxylapatite. Proteins were eluted with a linear KPO_4 buffer (pH 6.8) gradient in 10 mM 2-mercaptoethanol-10% glycerol. The 8-ml fractions were assayed for DNA supercoiling* (○) *and relaxation of native supercoiled ColE1 DNA-N-C (nicking-closing activity)* (△). *OD_{260}, Optical density at 260 nm.*

TABLE 1. *Sensitivity of DNA synthesis in toluene-treated cells to novobiocin and oxolinic acid*[a]

Cells	Incorporated [^{3}H]dTTP (pmol/10^8 cells per 30 min)			
	−ATP, −drug	+ATP, −drug	+ATP, +Nov	+ATP, +Oxo
B. subtilis 168 (wild type)	2.0	17.4	3.6	3.3
B. subtilis 168 (*trpC* Nov^r-O)	1.5	11.5	10.7	2.9
Nov^r-O transductant no. 1 Ade$^+$ (*novA*)	2.0	15.8	13.9	3.4
Nov^r-O transductant no. 2 Thr$^+$ (*novB*)	2.1	17.0	4.8	3.2
B. subtilis 168 (*trpC thy*)	1.8	13.8	4.8	2.6
B. subtilis 168 (*trpC thy* Nov^r-S)	1.4	11.9	9.7	2.5
B. subtilis 168 N10 (Nal^r)	2.2	12.0	3.4	12.6

[a] In vitro DNA synthesis in toluene-treated cells was measured in the absence or presence of 5 μg of novobiocin (Nov) per ml or 50 μg of oxolinic acid (Oxo) per ml, as described in the text. At the same time, the activity in the absence of rATP and the drugs was measured. The table shows the amount of [^{3}H]dTTP incorporated into acid-insoluble materials in a 30-min incubation at 30°C.

that DNA gyrase is a function essential for the replication of DNA in wild-type *B. subtilis* as well as in *E. coli* (3). However, mutants resistant to either novobiocin or nalidixic acid can incorporate [^{3}H]dTTP in the presence of the drug. *novA* but not *novB* strains are resistant to novobiocin, identifying *novA* as the target protein gene. Note that the reaction mixture contained 2 mM ATP, making estimates of the sensitivity of DNA synthesis to coumermycin or novobiocin artifically low, since these drugs act competitively with ATP (26; Sugino and Cozzarelli, in press; see below).

All of the *B. subtilis nal* mutations described to date have been mapped near *purA* at the replication origin of the chromosome (1a, 12, 31). The *nal* mutations of strain N-10, isolated in our laboratory, also were mapped in this region (Table 2). Linkage to genes outside of this region was not detected by using kit strains 2 through 9 (4). In confirmation of the results of previous workers (12, 13), the reciprocity and additivity of genetic recombination events in this region are anomolous (see Discussion). However, the *nalA* phenotype is extremely stable when expressed in transductants.

Two Nov^r mutants (Sueoka and Orrego) had mutations that were also shown to map in the same genetic region as reported earlier (31) (Table 3). Transductional linkage to the threonine locus, which is linked to *novB* (31), was also checked by using the Dedonder kit strain 8 (4). None of the more than 300 transductants from the Sueoka strain showed simultaneous acquisition of the Nov^r phenotype, whereas 60 of 120 Thr$^+$ transductants from the Orrego strain acquired the Nov^r phenotype. Similar experiments

TABLE 2. *Three-factor transduction crosses for mapping the resistance locus of strain N-10*[a]

Donor	Recipient	Selection (no.)	Recombinant class			No.
Nal^r N-10 (111)	Kit 1 *purA cys trp* (000)		Ade$^+$	Nal^r	Cys$^+$	
		Ade$^+$	1	0	0	206
		(386)	1	1	0	143
			1	1	1	17
			1	0	1	20
		Cys$^+$	0	0	1	268
		(479)	0	1	1	133
			1	1	1	36
			1	0	1	42
	JH855 *gua spo purA* (000)		Gua$^+$	Nal^r	Ade$^+$	
		Gua$^+$	1	0	0	64
		(142)	1	1	0	50
			1	1	1	15
			1	0	1	13
		Ade$^+$	0	0	1	137
		(240)	0	1	1	66
			1	1	1	31
			1	0	1	6

[a] Possible order: *ade nal gua cys.*

TABLE 3. *Three-factor transduction cross for mapping novobiocin resistance*[a]

Donor	Recipient	Selection (no.)	Recombinant class			No.
Nov^r-Orrego (111)	Kit 1 *purA cys trp* (000)		Cys$^+$	Nov^r	Ade$^+$	
		Ade$^+$	0	0	1	80
		(168)	0	1	1	54
			1	1	1	25
			1	0	1	9
		Cys$^+$	1	0	0	72
		(173)	1	1	0	66
			1	1	1	23
			1	0	1	12
Nov^r-Sueoka (111)	Kit 1 *purA cys trp* (000)		Cys$^+$	Nov^r	Ade$^+$	
		Ade$^+$	0	0	1	110
		(210)	0	1	1	71
			1	1	1	25
			1	0	1	4
		Cys$^+$	1	0	0	138
		(260)	1	1	0	76
			1	1	1	28
			1	0	1	18

[a] Order implied: *purA nov cys.*

showed other Nov^r strains in this lab also to be double mutants. These results suggest that the Orrego strain contained two lesions, *novA* and *novB*, whereas the Sueoka strain was solely *novA*.

The *novA* marker was transduced into kit strain 1 jointly with the *purA* marker, and *novB* was transduced into kit strain 8 jointly with the *thr* marker, for separate analyses of the in vitro resistance to novobiocin. The data suggest that *novA* is probably the only one of the two genes that directs the synthesis of a gyrase subunit.

Identification of genes controlling DNA gyrase in *B. subtilis*. To identify directly the genes of DNA gyrase, the enzyme activity was partially purified as described in Materials and Methods, and sensitivity to novobiocin (Fig. 2 and 3) and nalidixic acid (Fig. 3) was tested. DNA gyrase from wild-type *B. subtilis* is as sensitive to both drugs as is *E. coli* DNA gyrase (5, 7, 27). DNA gyrase from Nal^r *B. subtilis* N-10 was 50- to 100-fold more resistant to nalidixic acid than was DNA gyrase from wild-type cells (Fig. 3A). The DNA gyrase from a *novB* mutant was almost as sensitive as was the wild-type enzyme. However, DNA gyrase from a *novA* mutant was much more resistant to the drug than was the wild-type DNA gyrase (Fig. 2 and 3B). As shown, a very slight resistance of DNA gyrase from the *novB* mutant could be detected, but the enzyme from the *novA novB* double mutant had the same sensitivity as that from *novA* mutant (data not shown). If the *novA* and *novB* loci both conferred resistance to the gyrase, we would have detected an increased level of resistance in the double mutant; hence, it seems most likely that only the *novA* locus has a major affect on the function of the gyrase.

Therefore, we can conclude that DNA gyrase genes of *B. subtilis* are *nalA* and *novA*. However, we cannot exclude the possibility that additional gene products might be part of DNA gyrase in *B. subtilis*, since we have not yet purified the enzyme to homogeneity or succeeded in completely dissociating active subunits for reconstitution experiments.

Properties of DNA gyrase from *B. subtilis*. The partially purified DNA gyrase (fraction V) from wild-type *B. subtilis* 168 catalyzes several reactions, namely, DNA supercoiling, relaxation of DNA supercoiling, double-strand DNA cleavage inducted by nalidixic acid or oxolinic acid, and DNA binding similar to that of the *E. coli* enzyme (22). We found that the supercoiling reaction by the enzyme from *B. subtilis* also requires ATP and Mg^{2+} (no other nucleotide can replace ATP). The optimal concentration of Mg^{2+} was about 10 mM. The K_m

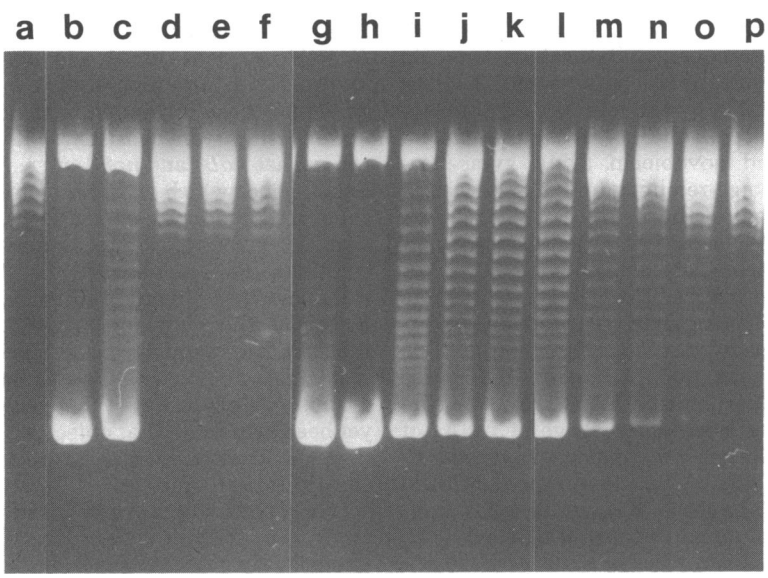

FIG. 2. *Sensitivity of DNA gyrases to novobiocin. The standard supercoiling assays contained 1 U of DNA gyrase from either B. subtilis 168 wild type, novA, or novB, 0.2 μg of relaxed ColE1 DNA, and the indicated amounts of novobiocin. After 60 min of incubation at 30°C, the products were analyzed by agarose gel electrophoresis. (a) Relaxed ColE1 DNA with no enzyme; (b) a + B. subtilis 168 DNA gyrase; (c) b + drug (0.04 μg); (d) b + drug (0.14 μg/ml); (e) b + drug (0.28 μg/ml); (f) b + drug (0.56 μg/ml); (g) a + novA DNA gyrase without novobiocin; (h) g + drug (0.014 μg/ml); (i) g + drug (0.14 μg/ml); (j) g + drug (0.28 μg/ml); (k) g + drug (0.56 μg/ml); (l) a + novB gyrase without novobiocin; (m) l + drug (0.014 μg/ml); (n) l + drug (0.14 μg/ml); (o) l + drug (0.28 μg/ml); (p) l + drug (0.56 μg/ml).*

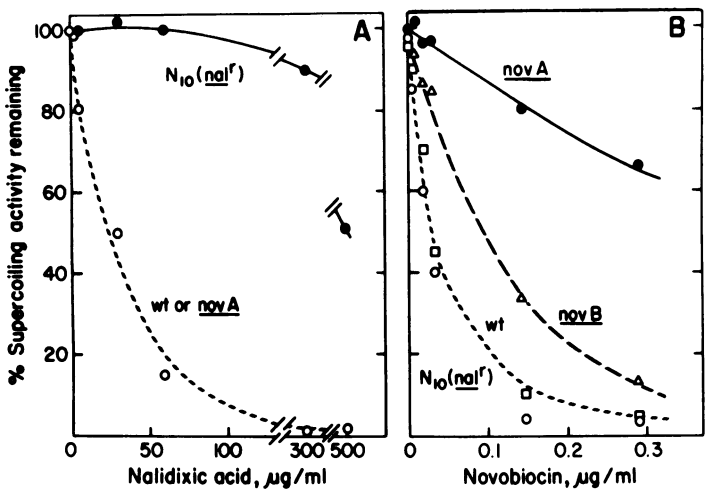

FIG. 3. *Sensitivity of DNA gyrase to nalidixic acid and novobiocin. Sensitivity was measured as for Fig. 2. (A) Sensitivity of DNA gyrase to nalidixic acid.* (○) *DNA gyrase from either B. subtilis 168 wild type (wt) or novA;* (●) *DNA gyrase from B. subtilis N-10 (Nal^r). (B) Sensitivity of DNA gyrase to novobiocin.* (○) *DNA gyrase from B. subtilis 168 wild type (wt);* (□) *DNA gyrase from B. subtilis N-10 (Nal^r);* (●) *DNA gyrase from B. subtilis novA;* (△), *DNA gyrase from B. subtilis novB.*

for ATP was 500 mM, and the K_m^{ap} for the relaxed ColE1 DNA was 30 µM. The activity was inhibited both by nalidixic acid and oxolinic acid and by novobiocin and coumermycin A_1 as mentioned above. The K_i values for nalidixic acid and novobiocin, measured as with the *E. coli* enzyme (26, 27), were 10 and 0.002 µM, respectively. Moreover, inhibition by novobiocin or coumermycin A_1 is competitive with ATP, as is the *E. coli* DNA gyrase (26).

In the absence of ATP, or in the presence of both ATP and novobiocin, DNA gyrase exhibited a breakage-rejoining (or nicking-closing) activity like that of the *E. coli* enzyme, and its activity was also sensitive to nalidixic acid but not to novobiocin (data not shown).

Oxolinic acid-induced DNA cleavage of DNAs by *B. subtilis* DNA gyrase was much less effective than that by *E. coli* DNA gyrase. The activity was about $\frac{1}{8}$ to $\frac{1}{10}$ of *E. coli* DNA gyrase at a similar stage of purity (Fig. 3). However, the favored cutting sites for breakage of DNAs of pCS540 (a chimeric *Staphylococcus aureus-E. coli* plasmid) (2), pMB9, p6 (plasmid pCS540 containing a 5-kilobase *B. subtilis* DNA fragment as a recombinant insert), ColE1, pBR322, λ, φX174RF, simian virus 40, and pUB110 (*B. subtilis* plasmid) are the same for the *B. subtilis* as for the *E. coli* gyrase (Fig. 4). This result emphasizes the importance of this specificity.

DISCUSSION

The present experiments demonstrated that, in addition to the organisms already characterized (3a, 6, 17, 22), *B. subtilis* also contains an active DNA gyrase. The fact that this enzyme showed a remarkable resemblance to its well-characterized counterparts from *E. coli* and *M. luteus* emphatically illustrates the importance of this enzymatic activity in widely divergent procaryotic species. At least two genes are now known to code for functional gyrase activity in *B. subtilis*. Genes *nalA* and *novA* have been known (1, 13), although until now no enzymatic defect has been attributed to them.

Our results were consistent with the interpretation that *nalA* and *novA* are separate, closely linked genes which code for distinctly different gene products essential for DNA gyrase function. There was no evidence suggesting any substantial difference between the *B. subtilis* enzyme and that already well characterized from *E. coli*. Although some physical separation of the *B. subtilis* subunits was achieved during this study (data not shown), the efficient dissociation of substrate quantities of these products has not yet been achieved. That objective is essential for the characterization of each protein and for reconstitution studies.

Our genetic studies were not intended to precisely map the *nal* or *nov* loci with respect to immediately adjacent genes, because that work is already known to be frought with difficulties (12, 13) and is the subject of extensive current investigation (28, 29). However, we did consider it essential to establish which loci were related to our biochemical analyses. The results clearly showed the involvement of *nalA* and *novA* but probably not of *novB*.

The *B. subtilis* genetic literature has consist-

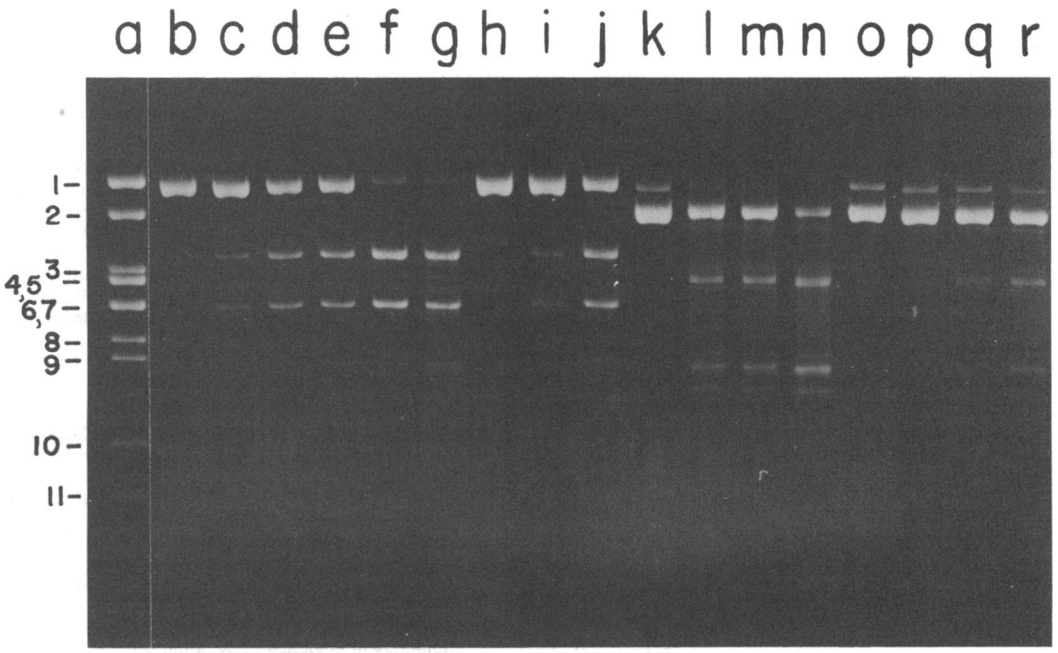

FIG. 4. *Comparison of DNA cleavage by E. coli and B. subtilis DNA gyrases. DNA cleavage induced by oxolinic acid was measured by using plasmid pMB9 and pCS540 DNAs treated with EcoRI. After 60 min of incubation with either E. coli or B. subtilis DNA gyrase at 30°C, the reaction was stopped with 1% sodium dodecyl sulfate, the mixture was treated with 50 μg of proteinase K per ml for 30 min at 37°C, and the DNA products were analyzed by electrophoresis in 1% agarose. Lane (a) is marker adenovirus type 2 DNA treated with HindIII. Fragment sizes were taken as (1) 9,190, (2) 5,050, (3) 3,310, (4) 3,170, (5) 3,100, (6) 2,720, (7) 2,610, (8) 2,195, (9) 2,020, (10) 1,290, and (11) 940 base pairs. (b) pCS540 DNA treated with EcoRI and no DNA gyrase. (c) b + 1 U of E. coli DNA gyrase. (d) b + 2.5 U of E. coli DNA gyrase. (e) b + 5 U of E. coli DNA gyrase. (f) b + 10 U of E. coli DNA gyrase. (g) b + 20 U of E. coli DNA gyrase. (h) b + 10 U of B. subtilis DNA gyrase. (i) b + 20 U of B. subtilis DNA gyrase. (j) b + 30 U of B. subtilis DNA gyrase. (k) pMB9 DNA treated with EcoRI and no DNA gyrase. (l) k + 5 U of E. coli DNA gyrase. (m) k + 10 U of E. coli DNA gyrase. (n) k + 20 U of E. coli DNA gyrase. (o) k + 5 U of B. subtilis DNA gyrase. (p) k + 10 U of B. subtilis DNA gyrase. (q) k + 20 U of B. subtilis DNA gyrase. (r) k + 30 U of B. subtilis DNA gyrase.*

ently pointed to these genes as difficult to characterize because of recombinational anomalies near their locus at the origin of chromosomal replication (12). In this study these problems are illustrated by the data of Tables 2 and 3. If one looks at the data in Table 2 as a simple two-factor cross, the recombination values clearly suggest that the order is Ade-Nal-Cys, but the three-factor recombinant classes do not confirm this. Similarly, in the cross among Gua^+ transductants, the frequency of recombinant classes is somewhat suspicious, whereas the frequency of Ade^+ transductants clearly suggests the proper order. Even in Table 3, where the results seem more straightforward, the double-crossover class still is slightly high relative to the other recombinants. Now that we know the biochemical alteration associated with these defects, it prompts a more thorough investigation to determine whether the anomalies are partially due to functional or dominance effects of the actual genes involved. Since these two genes are thought to be closely linked genetically, it will also be imperative to determine whether they are coordinately regulated, an unlikely probability in *E. coli*. Certainly the current studies suggest that the DNA gyrase is also essential for growth, DNA replication, and transcription of *B. subtilis* (8, 9, 21; D. Dubnau, personal communication), but as yet the extent to which it influences diverse cellular activities such as sporulation, germination, bacteriophage metabolism, plasmid replication, etc., is only speculative.

Purification of the *B. subtilis* DNA gyrase through DEAE-Sephacell and hydroxylapatite compared remarkably with that for the *E. coli* and *M. luteus* enzymes, yielding enzymes with quite similar structural properties and comparative specific activities. However, reconstitution or reassociation of enzyme subunits has not yet restored an activity comparable to that seen for *E. coli*.

We showed that DNA gyrase from wild-type *B. subtilis* shared with the *E. coli* and *M. luteus*

gyrases the same cofactor requirements for activity and the same sensitivity to a number of antibiotics. In fact, four of the five functions which characterize the *E. coli* enzyme (16, 22) were also possessed by the *B. subtilis* enzyme. ATPase activity has not yet been uniquely associated with the *B. subtilis* DNA gyrase, since the stage of enzyme purity obtained thus far still carries a residue of other cellular ATPase activities.

The similarity between properties of the *B. subtilis* enzyme and those of other gyrases was most remarkable with respect to oxolinic acid-induced DNA cleavage in the presence of sodium dodecyl sulfate. Although the specific activity for DNA cleavage by the *B. subtilis* enzyme was less than that characteristically observed for the *E. coli* enzyme, this reduced efficiency was also characteristic of the reconstituted *M. luteus* DNA gyrase (J. C. Wang, personal communication). The evidence strongly suggested that both enzymes recognized exactly the same sites on the DNA. In fact, with every DNA substrate tested, whether it originated from *E. coli*, *B. subtilis*, or another source, the specificities of cutting sites for breakage by *E. coli* gyrase and *B. subtilis* gyrase were almost identical—a result which, although not clearly understood, emphasized that gyrase binding to DNA was not random and must be fundamentally important to specificity.

Individual subunits of DNA gyrase from both *E. coli* and *M. luteus* have been purified and reconstituted in homologous and heterologous mixtures (1). Although no heterologous reconstitution was capable of restoring good DNA supercoiling activity or breakage-reunion activity, DNA cleavage activity could be restored. In fact, *E. coli* subunit B plus *M. luteus* subunit α reconstituted better cleavage activity than *M. luteus* subunits α and β. Moreover *Eco* topoisomerase III subunit ν (1) plus *M. luteus* gyrase subunit α also restored good cleavage activity. All restorations of cleavage activity indicated that the same recognition sites were being used as by homologous *E. coli* gyrase subunits. These results suggest that the recognition subunit of site specificity for DNA breakage is the A or α subunit, with the better efficiency dependent on *E. coli* subunits. Although we have not yet efficiently separated individual subunits of *B. subtilis* gyrase, some preliminary mixing experiments were done with individual subunits of *E. coli* DNA gyrase and a preparation of partially dissociated *B. subtilis* enzyme. Thus far, no complementation or stimulation of DNA gyrase reactions has been detected. This may reflect a stronger association of *B. subtilis* gyrase subunits and might also explain why we did not succeed in efficiently dissociating individual subunits during purification.

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