Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8860–8864, October 1991
Genetics

An unusual mechanism for resistance to the antibiotic coumermycin A_1

(coumarins/DNA topoisomerases/ATP-binding site)

IGNACIO DEL CASTILLO, JOSÉ LUIS VIZÁN, MARÍA DEL CARMEN RODRÍGUEZ-SÁINZ, AND FELIPE MORENO*

Unidad de Genética Molecular, Hospital Ramón y Cajal, Carretera de Colmenar km 9.100, Madrid, 28034, Spain

Communicated by Jon Beckwith, July 8, 1991

ABSTRACT **Bacterial DNA gyrases are type II topoisomerases made up of two A subunits and two B subunits. Coumarins are carbohydrate-containing antibiotics that inhibit topoisomerases II by competing with ATP for binding to the enzymes. High resistance to coumarins is produced in bacterial species by mutations in *gyrB*, the gene encoding subunit B. We have found an unusual mechanism of resistance to coumarins in *Escherichia coli*. This mechanism is exhibited by cells containing the wild-type *gyrB*, or its 5′ half, in high copy number. Since homologous mutant *gyrB* (coumermycin resistant) truncated genes did not confer drug resistance at all under the same conditions, we propose that this mechanism of resistance is due to drug sequestration by the overproduced wild-type GyrB polypeptides. A corollary of this is that the amino half of GyrB is required and sufficient to fashion the ATP-binding domain of DNA gyrase, a conclusion that was further supported by mapping three independent coumarin-resistant mutations at Arg-136 of GyrB. Just upstream of this residue there is a glycine-rich sequence highly conserved in all topoisomerases II, which seems to be a good candidate for the actual ATP-binding site.**

DNA topoisomerases are enzymes that catalyze a variety of interconversions between topological isomers of DNA (1–3). All known DNA topoisomerases relax closed circular DNA, but only bacterial topoisomerases II, usually known as DNA gyrases, have been shown to supercoil DNA. This supercoiling activity is achieved at the expense of ATP hydrolysis to ADP and phosphate (for reviews, see refs. 1, 4, and 5). In eubacteria, the ATP-independent relaxing activity of DNA topoisomerase I and the ATP-dependent DNA supercoiling activity of DNA topoisomerase II compete to produce the proper level of superhelical tension in the cell, which is important to biological functions such as DNA replication and transcription and certain types of genetic recombination (4, 6, 7).

The structural and biochemical functions of the *Escherichia coli* DNA gyrase have been extensively studied. This enzyme catalyzes negative DNA supercoiling in a process involving several steps—DNA binding, site-specific cleavage of both DNA strands and formation of transient covalent bonds between the 5′ termini of DNA and the enzyme, strand passage, and DNA resealing. This reaction is coupled to ATP hydrolysis. The enzyme is an A_2B_2 tetramer composed of two subunits, A and B (5, 8), which are respectively encoded by genes *gyrA* and *gyrB*, located at 48 and 83 min on the *E. coli* genetic map (9). Both genes have been sequenced and shown to encode proteins of 875 amino acids (97 kDa, subunit A) (10) and 804 amino acids (90 kDa, subunit B) (11, 12). Three classes of compounds are known to inhibit *E. coli* DNA replication by blocking DNA gyrase activity. Quinolones, such as nalidixic acid and oxolinic acid, act by trapping a gyrase–DNA reaction intermediate (13–15). The enterobacterial peptide microcin B17 acts in the same way (16). In contrast, coumarins, such as coumermycin A_1, novobiocin, and chlorobiocin, act by competing with ATP for binding to the enzyme (17). The fact that all the *E. coli* mutations conferring high resistance to coumarins have been mapped to *gyrB* strongly supports the theory that subunit B is the primary target of those drugs. As a corollary, this subunit has been thought to be responsible for the ATP-binding and ATP hydrolysis activities of the enzyme (4, 5). Indeed gyrase subunit B has been shown to exhibit a low level of ATPase activity (18, 19). By inhibiting the ATPase activity of DNA gyrase, the coumarins block the introduction of supercoils into relaxed DNA and relax supercoiled chromosomal DNA *in vivo* (20, 21). As a consequence, semiconservative DNA replication and cell growth are arrested.

In this paper we report an unusual mechanism of resistance to coumarins and provide significant data concerning the structure–function relationship of the *E. coli* GyrB protein.

MATERIALS AND METHODS

Media and Chemicals. Liquid and solid LB-rich media and M63 minimal medium were prepared as in ref. 22. Antibiotics were added to plates at the following final concentrations: ampicillin, 30 μg/ml; tetracycline, 20 μg/ml; kanamycin, 30 μg/ml; coumermycin A_1, 16 μg/ml.

Genetic Techniques and DNA Manipulations. Bacterial cultures, phage lysates, and generalized P1 transduction were carried out as described (22). All DNA manipulations, including restriction enzyme digestions, ligations, transformations, and agarose gel electrophoresis, were performed as indicated (23).

Bacterial Strains. The relevant characteristics of the *E. coli* K-12 strains used in this work are as follows: pop3351 (*recA*$^+$ *gyrB*$^+$), RYC1000 (*recA56 gyrB*$^+$), and RYC1010 [*recA56* (λ *precA*$^+$ *c*Iind) *gyrB*$^+$, a RYC1000 derivative]. RYC1020 (*gyrB320*), RYC1021 (*gyrB321*), and RYC1022 (*gyrB322*) are three spontaneous mutants from RYC1010 selected for their ability to grow on M63 plates supplemented with coumermycin A_1 at 16 μg/ml. RYC1030 (*gyrB320 gyrB301 zid*::Tn*10*) is a derivative of RYC1020 obtained by transducing via P1 the other markers [*gyrB301* is a mutation conferring resistance to the peptide antibiotic microcin B17 (16)]. LE316 [*gyrB*(ts)] was obtained from E. Orr (24); it was described as chlorobiocin resistant; this strain is coumermycin A_1 sensitive (Cou^s) at 30°C in our hands. PolA$^-$ derivatives from these strains were obtained by introducing the *polA5* allele with a linked Tn*10* by P1 transduction. Strain 71.18 [Δ(*lac-proAB*) *supE thiA* (F′ *proAB lacI*^{q} *lacZ*ΔM15)] was used to propagate

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Abbreviations: MIC, minimal inhibitory concentration; Cou^r and Cou^s, coumermycin resistant and sensitive, respectively; Ap^r, ampicillin resistant; PLP-AMP, pyridoxal-5′-diphospho-5′-adenosine.

*To whom reprint requests should be addressed.

M13 and to obtain single-stranded DNA for the sequencing reactions.

Plasmids. The structures of pBR322 plasmid derivatives used in this work are shown in Fig. 1. pCID500, pCID509, and pCID510 were obtained in a previous study (16). All three contain a 8.5-kilobase (kb) *Bam*HI fragment from *E. coli*, which includes genes *recF* and *gyrB* and adjacent sequences from either RYC1010 (pCID510) or RYC1030 (pCID500 and pCID509). The other plasmids (except pCID549) were obtained by *in vitro* deletion of different fragments from the above plasmids (see Fig. 1); series 501–504 derive from pCID500 (*gyrB301 gyrB320*), and series 511–514 derive from pCID510 (*gyrB*$^+$).

pCID549 was obtained as follows. Total chromosomal DNA from pop3351 was digested to completion with *Eco*RI. The resulting DNA fragments were separated by agarose gel electrophoresis and those of 6–8 kb were extracted and ligated into the *Eco*RI site of plasmid pCID509.8 (Fig. 1). The ligation mixture was used to transform LE316 [*gyrB*(ts)] (Cou^s), and ampicillin-resistant (Ap^r) clones growing at the restrictive temperature (42°C) were selected. pCID507 and pCID517 were obtained by cloning the 8.5-kb DNA chromosomal fragment from pCID500 and pCID510 into the *Bam*HI site of the oligocopy (six to eight copies per cell) plasmid pLG339 (25). pMK47 is a pKC16 derivative that carries the wild-type *gyrB* gene from a strain unrelated to ours (26).

Cloning by *in Vivo* Allelic Replacement. To clone the *gyrB* genes from the coumermycin A_1-resistant (Cou^r) strains RYC1021 and RYC1022, we used the method described in ref. 27. This method takes advantage of the fact that plasmids using the ColE1 replicon, such as pBR322, cannot be maintained in a *polA* background, unless they become inserted in the chromosome by a homologous recombination event. The *polA5* marker was P1 transduced into strains RYC1021 and RYC1022 as indicated above. The resulting strains were transformed with plasmid pCID510.1, which contains only 68 nucleotides from the 5′ end of *gyrB* (Fig. 1). P1 lysates prepared on these transformant strains were used to infect the *polA*$^+$ parental strains, and Ap^r clones were selected. In the *polA*$^+$ background, the plasmid inserted in the chromosome will be resolved by homologous recombination and replicate autonomously. Plasmid DNA was prepared from a pool of Ap^r clones and used to transform strain LE316 [*gyrB*(ts)]. The Ap^r transformants able to complement the *gyrB*(ts) mutation at the restrictive temperature (42°C) were selected, and their plasmids were analyzed by digestion with restriction endonucleases. In each case, they contained the *gyrB* allele of the parental Cou^r strains; pCID547 harbored *gyrB321* and pCID548 harbored *gyrB322*.

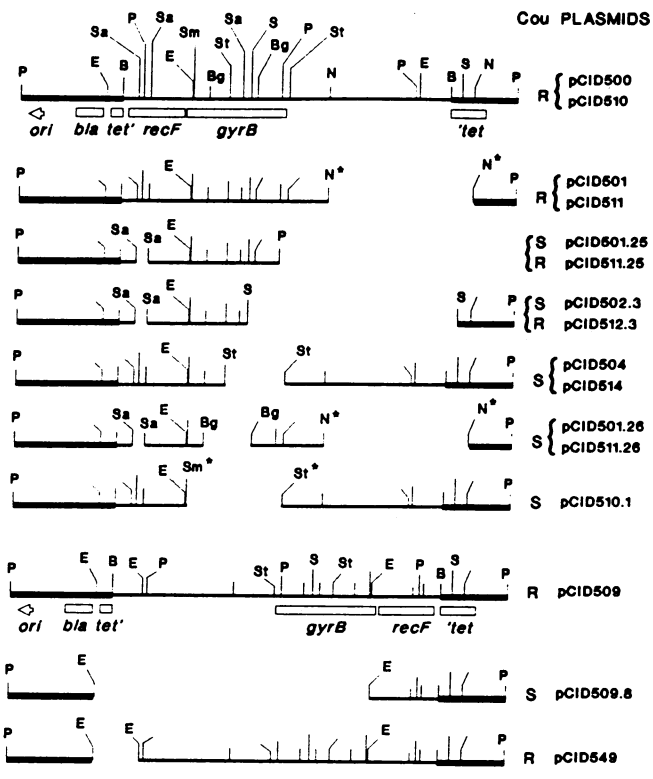

FIG. 1. Physical maps and relevant characteristics of plasmids used in this work. The coumermycin phenotype that each plasmid confers to *gyrB*$^+$ (Cou^s) cells in the presence of coumermycin at 16 μg/ml is indicated in the "Cou" column. Thick lines indicate sequences from pBR322, and thin lines indicate chromosomal sequences. B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; N, *Nru* I; P, *Pvu* II; S, *Sal* I; Sa, *Sac* II; Sm, *Sma* I; St, *Stu* I.

Detection of Plasmid-Encoded Polypeptides. Strain RYC1000 harboring plasmids to be analyzed was used to prepare maxicells as described (28). Labeling with 50 μCi (1 Ci = 37 GBq) of [^{35}S]methionine per ml was performed for 3 min at 37°C. One-half of each culture (0.5 ml) was immediately processed, and the other half was incubated for 30 additional min in medium containing a large excess of unlabeled methionine (250 μg/ml). Labeled proteins were separated by SDS/PAGE as described (29).

DNA Sequence Analysis. Each of the *Eco*RI–*Bgl* II fragments of the 5′ end of *gyrB* from plasmids pCID500, pCID510, pCID547, and pCID548 was purified from polyacrylamide gels and digested with *Sau*3A. Each one yielded the two expected fragments, an *Eco*RI–*Sau*3A fragment of 272 base pairs and a *Sau*3A–*Bgl* II fragment of 150 base pairs. The larger fragment was subcloned between the *Eco*RI and *Bam*HI sites of M13mp18 and M13mp19, and the smaller fragment was cloned into the *Bam*HI site of M13mp18 in the two possible orientations. The nucleotide sequence of both strands of each fragment was determined by the dideoxynucleotide chain-termination method (30).

Minimal Inhibitory Concentration (MIC) Determination. The minimal antibiotic concentration inhibiting growth for each strain was determined on multiwell plates containing M63 liquid medium and serial 2-fold dilutions of coumermycin A_1. Bacteria cultured overnight in M63 medium were diluted and added to wells at 2×10^6 colony-forming units per ml. The MICs were determined after incubation for 24 h at 37°C. The values are the means of three independent experiments.

RESULTS

The Wild-Type *gyrB* Allele Confers Resistance to Coumermycin A_1 when Cloned into a Multicopy Plasmid. We cloned into pBR322 the *gyrB* genes from the isogenic strains RYC1010 [*gyrB*$^+$ (Cou^s)] and RYC1030 [*gyrB301 gyrB320* (Cou^r)], obtaining plasmids pCID510 and pCID500, respectively (Fig. 1) (16). When RYC1010 was transformed with these plasmids, all the Ap^r transformants obtained were Cou^r (they grew on M63 plates supplemented with coumermycin A_1 at 16 μg/ml, a concentration that inhibits the growth of the sensitive strain RYC1010). Whereas the result obtained with pCID500 (*gyrB320*) might be expected, that obtained with pCID510 (*gyrB*$^+$) was not. To rule out the possibility that the Cou^r phenotype conferred by plasmid pCID510 to Cou^s cells was due to a putative *gyrB*(Cou^r) mutation silent in a single copy but expressed in high numbers of copies, we cloned the *gyrB*(Cou^s) gene from pop3351, a strain not related to RYC1010 (see *Materials and Methods*). The resulting plasmid, pCID549, also conferred coumermycin A_1 resistance to RYC1010 and other Cou^s strains. The same occurred with pMK47, a high-copy-number plasmid containing *gyrB* from another source (26). Since *gyrB* alleles from different Cou^s

strains yielded the same result, we concluded that the wild-type *gyrB* gene in high copy number determines resistance to coumermycin A_1.

Truncated *gyrB* Genes also Confer Resistance to Coumermycin A_1. One explanation for the Cou^r phenotype conferred by *gyrB*$^+$ on a high-copy-number plasmid is that coumermycin A_1 binds to and is sequestered by the excess GyrB. To identify the GyrB fragment containing this coumermycin A_1 binding site, we constructed several deletion derivatives from pCID510 and tested their ability to confer coumermycin A_1 resistance. We first constructed plasmid pCID511.25 by eliminating the 3′ end of *gyrB*$^+$ (removal of the *Pvu* II fragment, Δ*Pvu*II) (Fig. 1). As expected, this plasmid was unable to complement the *gyrB*(ts) mutation of strain LE316 for growth at 42°C, but it retained the ability to confer coumermycin A_1 resistance at 30°C. In other words, the elimination of the last 168 nucleotides of the 3′ end of *gyrB*$^+$ did not affect the ability of this gene to confer resistance. We also constructed pCID501.25 from pCID500 (*gyrB320*). In this case, the removal of the *Pvu* II fragment caused the loss of the resistance determined by the entire gene. These results strongly support the idea that the drug sequestration is the molecular basis of the resistance we observed in the wild-type cells overproducing the wild-type GyrB polypeptide. They further indicate that the mutated polypeptide GyrB320 has lost affinity for coumermycin A_1.

To determine if shorter fragments of the 5′ end of *gyrB* retained the ability to protect cells from coumermycin A_1, we constructed the 3′ *Sal* I (Δ*Sal*I) and 3′ *Stu* I (Δ*Stu*I) deletions, as well as the internal *Bgl* II (Δ*Bgl*II) deletion of *gyrB*, as shown in Fig. 1. The 3′ *Sal* I *gyrB* deletion derivative containing wild-type sequences (pCID512.3) still conferred coumermycin A_1 resistance. However, neither the 3′ *Stu* I deletion derivative (pCID514) nor the internal *Bgl* II deletion derivative (pCID511.26) conferred any resistance. As expected, none of the homologous derivatives carrying sequences from the mutated gene conferred antibiotic resistance to Cou^s cells. It must be noted that stable truncated GyrB polypeptides with the expected size were produced from all these (wild-type and mutant) genes in maxicells: 84 kDa from Δ*Pvu*II, 55 kDa from Δ*Sal*I, 43 kDa from Δ*Bgl*II, and 36.5 kDa from Δ*Stu*I (Fig. 2). The above results indicated that the *gyrB* sequence upstream of nucleotide 1501 (*Sal* I site) is sufficient to protect cells from coumermycin and that part or all of the sequence between nucleotide 953 (*Stu* I site) and nucleotide 1501 is required for this protection to be efficient. In addition, these results indicated that the *gyrB320* mutation should be located upstream of the *Sal* I site.

Three Independent Mutations Conferring Resistance to Coumermycin A_1 Affect the Same Codon of *gyrB*. The above results allow us to distinguish plasmids carrying wild-type *gyrB* sequences from those carrying Cou^r mutations, which therefore opens the possibility of mapping coumermycin A_1 resistance mutations to specific fragments within *gyrB*. To map *gyrB320*, we exchanged homologous restriction fragments (*Bgl* II and *Stu* I) between pCID501.2 and pCID511.2 (Fig. 1). Then we eliminated the 3′ end of the hybrid *gyrB* genes (Δ*Pvu*II) and tested the ability of these derivatives to confer coumermycin A_1 resistance to wild-type cells. Our results clearly indicated that the mutation *gyrB320* was located near the 5′ end of *gyrB*, upstream from the *Bgl* II site at position 428.

Then we cloned two other mutations (*gyrB321* and *gyrB322*) from independent spontaneous Cou^r mutants of RYC1010. This was done by the P1-mediated gene replacement procedure (27), using as cloning vector the plasmid pCID510.1 (Fig. 1). As expected, the resulting plasmids (pCID547 and pCID548, respectively) conferred coumermycin resistance to Cou^s cells but lost this property after elimination of their 3′ *Pvu* II fragments.

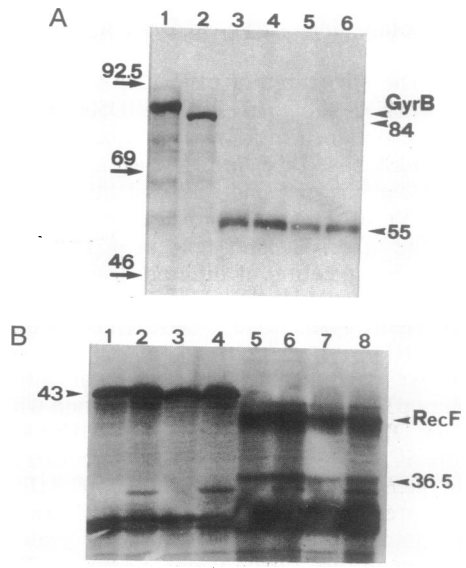

FIG. 2. Plasmid-encoded polypeptides in maxicells. (*A*) Lanes: 1, pCID511.2 (*gyrB*$^+$); 2, pCID511.25 (Δ*Pvu*II); 3 and 5, pCID502.3 (Δ*Sal*I); 4 and 6, pCID512.3 (Δ*Sal*I). Lanes 5 and 6 represent the pulse–chase experiments. (*B*) Lanes: 1 and 2, pCID511.26 (Δ*Bgl*II); 3 and 4, pCID501.26 (Δ*Bgl*II); 5 and 6, pCID514 (Δ*Stu*I); 7 and 8, pCID504 (Δ*Stu*I). Lanes 1, 3, 5, and 7 represent the pulse–chase experiments. Numbers indicate the size (in kDa) of the different polypeptides and molecular size standards.

Based on the assumption that the *gyrB321* and *gyrB322* mutations were located close to *gyrB320*, we sequenced the 422-base-pair *Eco*RI–*Bgl* II fragments from pCID500, pCID510, pCID547, and pCID548 on both strands by the dideoxynucleotide chain-termination method. The sequence we determined from pCID510 (wild-type *gyrB*) was identical to that previously published (11, 12). Mutations *gyrB320* and *gyrB322* were both due to a G·C → T·A transversion at position 407, which results in the replacement of Arg-136 by Leu in the amino acid sequence of the GyrB polypeptide. The *gyrB321* mutation was a C·G → T·A transition at position 406, which results in the change of Arg-136 → Cys.

The Level of Resistance to Coumermycin A_1 Depends on the Gene Dose. To estimate the degree of resistance conferred by mutations and plasmids carrying *gyrB* sequences, we determined the MIC of each strain. As shown in Table 1, the resistance to coumermycin A_1 was at least 32-fold higher in our *gyrB* mutants than in wild-type cells and 8-fold higher in cells harboring many copies of entire *gyrB* genes. This level of resistance was maintained by the deleted derivatives lacking the 3′ end (Δ*Pvu*II and Δ*Sal*I) of the otherwise wild-type genes, but no resistance was conferred by the corresponding deleted mutant derivatives. Cells carrying the wild-type *gyrB* gene cloned in an oligocopy vector (plasmid pCID517) were only 2-fold more resistant than wild-type cells. As would be expected, this suggests that the resistance mediated by drug sequestration is dependent on the *gyrB* gene dose. On the other hand, it should be noted that entire cloned genes, wild-type and mutant, conferred similar levels of coumermycin A_1 resistance to Cou^s wild-type cells, a result discussed below.

DISCUSSION

The results described above reveal an unusual mechanism of resistance to coumarins and provide additional information on the structure–function relationship in DNA gyrase. It is generally accepted that the competitive inhibition of DNA gyrase by coumarins can be prevented by mutations in *gyrB*. The mutant B subunit would lose affinity for the drug while

Table 1. MIC of coumermycin A_1 for wild-type (wt) and mutant *E. coli* strains

Strain	MIC, μg/ml
RYC1000 (wt *gyrB recA56*)	5
RYC1010 (wt *gyrB*)	5
RYC1020 [*gyrB320*(Cou^r)]	160
RYC1021 [*gyrB321*(Cou^r)]	320
RYC1022 [*gyrB322*(Cou^r)]	160
RYC1010 (pCID517) (wt *gyrB*, oligocopy)	10
RYC1010 (pCID507) [*gyrB320*(Cou^r), oligocopy]	40
RYC1010 (pCID510) (wt *gyrB*, multicopy)	40
RYC1010 (pCID500) [*gyrB320*(Cou^r), multicopy]	40
RYC1010 (pCID511.25) (wt *gyrB* Δ*Pvu*II, multicopy)	40
RYC1010 (pCID501.25) (*gyrB320* Δ*Pvu*II, multicopy)	5
RYC1010 (pCID512.3) (wt *gyrB* Δ*Sal*I, multicopy)	20
RYC1010 (pCID502.3) (*gyrB320* Δ*Sal*I, multicopy)	5
RYC1010 (pCID514) (wt *gyrB* Δ*Stu*I, multicopy)	5
RYC1010 (pCID504) (*gyrB320* Δ*Stu*I, multicopy)	5
RYC1010 (pCID511.26) (wt *gyrB* Δ*Bgl*II, multicopy)	5
RYC1010 (pCID501.26) (*gyrB320* Δ*Bgl*II, multicopy)	5

MICs of novobiocin for RYC1010 (wt *gyrB*) and RYC1020 (*gyrB320*) were 0.5 and >4 mg/ml, respectively.

retaining its ability to bind ATP efficiently enough to yield an active DNA gyrase. We show here that resistance to coumermycin can be provided by increasing the gene dose per cell of the wild-type *gyrB*(Cou^s) allele and that this resistance is maintained when different fragments of its 3′ end are eliminated. Given that deletion derivatives with the same structure from mutant *gyrB*(Cou^r) genes did not confer any antibiotic resistance at all, we propose that this mechanism of resistance to coumarins lies in the ability of the GyrB subunit to bind the drug. Overproduction of polypeptides containing the unaltered binding site would result in the titration of the antibiotic and, as a consequence, antibiotic-free DNA gyrase complexes could bind ATP and be functional. In contrast, overproduction of truncated mutant GyrB (Cou^r) polypeptides should produce no depletion of the intracellular concentration of antibiotic and, consequently, no effect on the susceptibility of cells to the antibiotic (Table 1). Further support for this view was provided by the fact that the level of antibiotic resistance was dependent on the *gyrB* gene dose and by the fact that the three Cou^r mutations that we have characterized map to the *gyrB* region shown to be involved in the sequestration phenomenon (i.e., the amino half of the protein).

A polypeptide containing the first 500 amino residues of GyrB (Δ*Sal*I) is apparently as efficient as the entire polypeptide in antibiotic sequestration. The fact that a polypeptide containing the first 318 residues (Δ*Stu*I) did not confer any resistance to coumermycin A_1 indicates that part or all of the polypeptide segment extending from residue 319 to residue 500 (*Sal* I site) is required for coumarin titration. On the other hand, because the residue altered in our mutants (Arg-136) maps 180 residues upstream of the *Stu* I site, it may be concluded that most of the amino half of GyrB, if not all, is required for the correct conformation of the coumarin-binding site.

In addition to DNA gyrase, another topoisomerase II (topo II′) has been isolated from *E. coli* (31, 32). This one, also a tetramer, consists of two GyrA subunits and two copies of a 50,000-kDa polypeptide called *v*. This polypeptide has been shown to be the carboxyl-terminal fragment of GyrB beginning at position 394 (12) (Fig. 3). Topo II′ can relax both negatively and positively supercoiled DNA (ATP-independent processes) but cannot supercoil DNA. Indeed topo II′ has no ATPase activity. Based on these facts, two independent functional domains were proposed for the GyrB polypeptide (12, 31). The carboxyl-terminal domain (fragment *v*, present in topo II′), with the ability to bind and stimulate GyrA to cut and reseal DNA, and the amino domain (absent in topo II′), with the ability to bind and possibly to hydrolyze ATP. Two predictions may be made from this hypothesis. The first one is that *gyrB* mutations conferring resistance to agents trapping the DNA breaking–rejoining activity of DNA gyrase should map within the carboxyl-terminal domain. Indeed, this is the case for the four known mutations of this type, two quinolone-resistance mutations (11) and two microcin B17-resistance mutations (16). The second one is that mutations conferring resistance to compounds that inhibit the binding of ATP to the B subunit, such as Cou^r mutations, should map within the amino-terminal domain, which is shown here for three mutations. Since coumarins competitively block the access of ATP to its binding site on the B subunit (17), all our results strongly support the idea that the amino domain of GyrB actually contains the nucleotide-binding site.

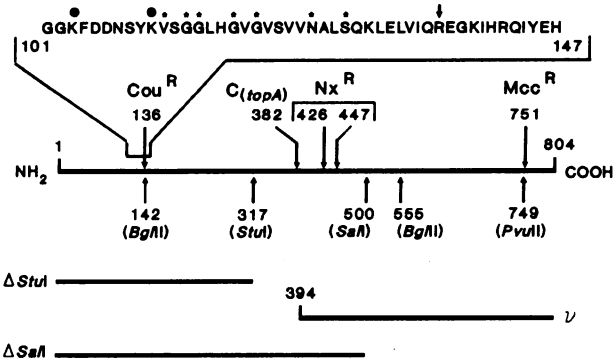

FIG. 3. Structure of the *E. coli* GyrB polypeptide and some of its truncated derivatives. Amino acid residues are indicated by numbers. The locations of the different mutations hitherto characterized are shown on the top of the figure. These include *gyrB301*, conferring resistance to the antibiotic microcin B17 (Mcc^r; Trp-751 → Arg) (16); *nal-24* and *nal-31*, conferring resistance to nalidixic acid (Nx^r; Asp-426 → Asn and Lys-447 → Glu, respectively) (11); *gyrB225*, compensatory mutation of *topA* deletions (duplication of a 6-base-pair sequence encoding Ala-Arg at position 382) (33); and the Cou^r mutations described in this work (*gyrB320*, Arg-136 → Leu and *gyrB321*, Arg-136 → Cys). The amino acid sequence of the region where the Cou^r mutations are located is also shown. An arrow points to Arg-136 and bold circles indicate Lys residues marked with PLP-AMP (34). Asterisks indicate residues highly conserved in the nine topoisomerases II whose sequences are known.

The fact that all three Cou^r mutations at Arg-136 resulted in high-level resistance to coumermycin A_1 (i.e., an important loss of affinity for the drug) indicates that this residue is essential for coumermycin binding, although it does not seem to be directly involved in ATP-binding because the mutant cells grew normally (i.e., they have an active gyrase). Genes encoding eight type II topoisomerase enzymes and the *gyrB* gene of *Pseudomonas putida* have been sequenced (35–39). The deduced amino acid sequences share extensive homology with one another. Whereas the amino-terminal part of the eukaryotic enzymes is related to bacterial gyrase B subunits, the rest of their sequences is similar to gyrase A subunits (35). In spite of these homologies, unambiguous ATP-binding consensus regions (as defined in refs. 40 and 41) have not been identified in these enzymes. Among the many ATP-requiring enzymes, only topoisomerases II and the vaccinia virus type I topoisomerase are known to be inhibited by coumarins at low concentration (reviewed in ref. 42). This implies that these enzymes may have an atypical ATP-binding site. On the other hand, the small amount of structural homology shared by ATP and the coumarins raises the question whether coumarins and ATP bind to the same site

on the *E. coli* gyrase B subunit (17). Whether or not this is the case, the short highly conserved region located just upstream of Arg-136 seems the best candidate to be the ATP-binding site on the B subunit (Fig. 3). In addition to its proximity to Arg-136, this sequence is rich in glycines, a feature found in ATP-binding sequences of many proteins (40, 41). These glycine-rich sequences have been proposed to function as "flexible loops," with the ability to promote conformational changes leading to the interaction of protein catalytic residues with the phosphoryl groups of bound ATP (43). In fact, lysyl residues within these structures have been shown to be specifically modified by ATP affinity analogs such as pyridoxal-5′-diphospho-5′-adenosine (PLP-AMP) in well-characterized ATPases (44–47).

The results in Table 1 merit some comments. Mutant cells exhibited a high resistance to coumermycin A_1. However the wild-type cells overproducing mutated GyrB polypeptides were 8-fold less resistant (Table 1, rows 7 and 9), a figure similar to that obtained by overproducing wild-type GyrB polypeptides. This apparently paradoxical result may be interpreted as follows. In *E. coli* cells overproducing mutated GyrB polypeptides, most DNA gyrase tetramers would contain two mutated B subunits and, consequently, should not bind coumermycin. The other tetramers (10–20%), containing at least one wild-type subunit, should be susceptible to drug inactivation. Nonfunctional (drug-blocked) tetramers should compete with the functional ones (drug-resistant) to occupy the DNA gyrase-binding sites on the bacterial chromosome. So the relative amount of nonfunctional drug-sensitive tetramers in the tetramer cell pool must be the determining factor in cell susceptibility to coumermycin A_1.

While this paper was in preparation, we learned of the existence of two other groups reporting results consistent with ours (34, 48). The first of these presents the nucleotide sequences of the wild-type *gyrB* gene and of a novobiocin-resistant *gyrB* allele from the halophilic archaebacterium *Halopherax*. The wild-type sequence is very similar to *gyrB* from other bacteria and may be aligned with them by hand. The mutant gene exhibits the three following changes: Asp-82 → Gly, Ser-121 → Thr, and Arg-136 → His (position numbers are those of *E. coli*). Of these changes, presumably only the third one causes drug resistance. In effect, Asp-82, also present in GyrB of *P. putida* (39) and ParE of *E. coli* (38), is just replaced by Gly in *E. coli* and *Bacillus subtilis* GyrB subunits. Amino acids at positions 121 and 136 are the same in all bacterial GyrB subunits. Since the change Ser-121 → Thr is conservative, Arg-136 → His remains as the unique significant change in the mutant protein. More important are the results described by Tamura and Gellert (34). They have shown that Lys-103 and Lys-110 of the B subunit of *E. coli* are specifically marked with radioactive PLP-AMP. All these results confirm that the amino half of GyrB contains the functional domain of the prokaryotic gyrases binding coumarins and ATP. These results also indicate that the glycine-rich sequence shown in Fig. 3 is directly involved in the binding of these compounds.

It may be noted that, like GyrB, GyrA has been shown to consist of two domains. A 64-kDa fragment derived from the amino terminus is sufficient to catalyze DNA supercoiling in the presence of the B subunit. The remaining 33-kDa fragment is presumed to contribute to the stability of the gyrase–DNA complex (49).

We thank C. Martín, J. Talavera, and A. Valero for technical assistance. This research was supported by a grant from Comisión Interministerial de Ciencia y Tecnología (Spain). I.d.C. was a recipient of a Caja Madrid fellowship. M.C.R.-S. and J.L.V. were recipients of Fondo de Investigaciones Sanitarias de la Seguridad Social fellowships.

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