

## Construction and Characterization of Mutations at Codon 751 of the *Escherichia coli gyrB* Gene That Confer Resistance to the Antimicrobial Peptide Microcin B17 and Alter the Activity of DNA Gyrase

FRANCISCO J. DEL CASTILLO, IGNACIO DEL CASTILLO, AND FELIPE MORENO\*

*Unidad de Genética Molecular, Hospital Ramón y Cajal, 28034-Madrid, Spain*

Received 11 October 2000/Accepted 18 December 2000

**Microcin B17 is a peptide antibiotic that inhibits DNA replication in *Escherichia coli* by targeting DNA gyrase. Previously, two independently isolated microcin B17-resistant mutants were shown to harbor the same *gyrB* point mutation that results in the replacement of tryptophan 751 by arginine in the GyrB polypeptide. We used site-directed mutagenesis to construct mutants in which tryptophan 751 was deleted or replaced by other amino acids. These mutants exhibit altered DNA gyrase activity and different levels of resistance to microcin B17.**

Type II DNA topoisomerases are enzymes that alter the topological state of DNA molecules by catalyzing strand transfer through transient, double-stranded breaks (24). Among them, bacterial DNA gyrase is the only topoisomerase known to be able to add negative supercoils to covalently closed circular DNA (11). Thanks to this unique property, DNA gyrase contributes to maintaining the supercoiling level required for bacterial DNA replication, transcription, and recombination (8, 24). The reaction involves the formation of a covalent protein-DNA intermediate and is coupled to the hydrolysis of ATP. *Escherichia coli* DNA gyrase is an A<sub>2</sub>B<sub>2</sub> tetramer. The site for DNA breakage and rejoining is located in the A subunit, encoded by the *gyrA* gene, whereas the site for ATP binding and hydrolysis lies in the B subunit, encoded by the *gyrB* gene (19).

DNA gyrase is essential for cell viability, and therefore it is a prime target for antibacterial therapy. There are two classes of DNA gyrase inhibitors, those that compete with ATP for binding to the enzyme (e.g., coumarins), and those that act by trapping the gyrase-DNA reaction intermediate, the so-called cleavable complex (e.g., quinolones) (9, 17).

Microcin B17 (MccB17) is a hydrophobic peptide antibiotic that is active against most enterobacteria (2) and inhibits DNA gyrase by trapping the gyrase-DNA cleavable complex (23). As a result, DNA replication is blocked, the SOS response is induced, and massive degradation of DNA ensues (14). MccB17 is produced by *Escherichia coli* cells that harbor the plasmid-borne *mcb* operon (13, 22). The antibiotic is synthesized in the ribosome as a 69-amino-acid precursor (5) that undergoes two steps of posttranslational modification, the formation of eight heterocyclic rings (four oxazole and four thiazole rings), and the cleavage of a 26-amino-acid-long leader peptide (27).

In a previous work, two MccB17-resistant mutants were isolated independently by unrelated *in vivo* methods. Interestingly, both mutants carried the same AT→GC transition at position 2251 of the *gyrB* gene, which results in the replacement of tryptophan 751 by arginine (W751R) in the C terminus of the GyrB polypeptide (23). To investigate the role of this residue in the resistance to Mcc B17 and in the activity of DNA gyrase, we used site-directed mutagenesis to generate an in-frame deletion of this codon and to replace tryptophan 751 (W751) with other amino acids.

**Site-directed mutagenesis of codon 751 of *E. coli gyrB*.** To obtain a template for site-directed mutagenesis, we amplified by PCR a 988-bp DNA fragment containing the 3' end of the *E. coli* K-12 *gyrB* gene. This fragment stretches from the unique intragenic *SalI* site to the extragenic *StuI* site located downstream of *gyrB* (Fig. 1). For subsequent manipulation, we added a *SalI* site to the sequence of the lower primer that we used. The PCR fragment was cloned into the pMOSBlue phagemid T-vector (recombinant plasmid pCFP490) and sequenced to confirm that no artifactual mutations were introduced during PCR amplification.

Site-directed mutagenesis was performed by the Kunkel method (16), using plasmid pCFP490 as the template. We replaced the W751 residue with lysine (W751K), glutamic acid (W751E), phenylalanine (W751F), glycine (W751G), and histidine (W751H). In addition, we also constructed an in-frame deletion of the W751 residue ( $\Delta$ W751). All plasmid inserts were sequenced after mutagenesis to confirm that the intended mutation had indeed taken place and that undesired mutations were absent.

The DNA fragments containing the desired mutations were recovered by *SalI* digestion of pCFP490 derivatives and cloned in the unique *SalI* site of plasmid pCID503 (7) (Fig. 1), which contains the 5' end of *gyrB*, to assemble a complete, mutated *gyrB* gene (recombinant plasmids pCFP501K, pCFP501E, pCFP501F, pCFP501G, pCFP501H, and pCFP501 $\Delta$ W).

**Mutation  $\Delta$ W751 abolishes GyrB function.** We tested whether the mutant *gyrB* genes encoded functional GyrB

\* Corresponding author. Mailing address: Unidad de Genética Molecular, Hospital Ramón y Cajal, Carretera de Colmenar Km 9, 28034-Madrid, Spain. Phone: 34-91-3368541. Fax: 34-91-3369016. E-mail: fmoreno@hrc.insalud.es.

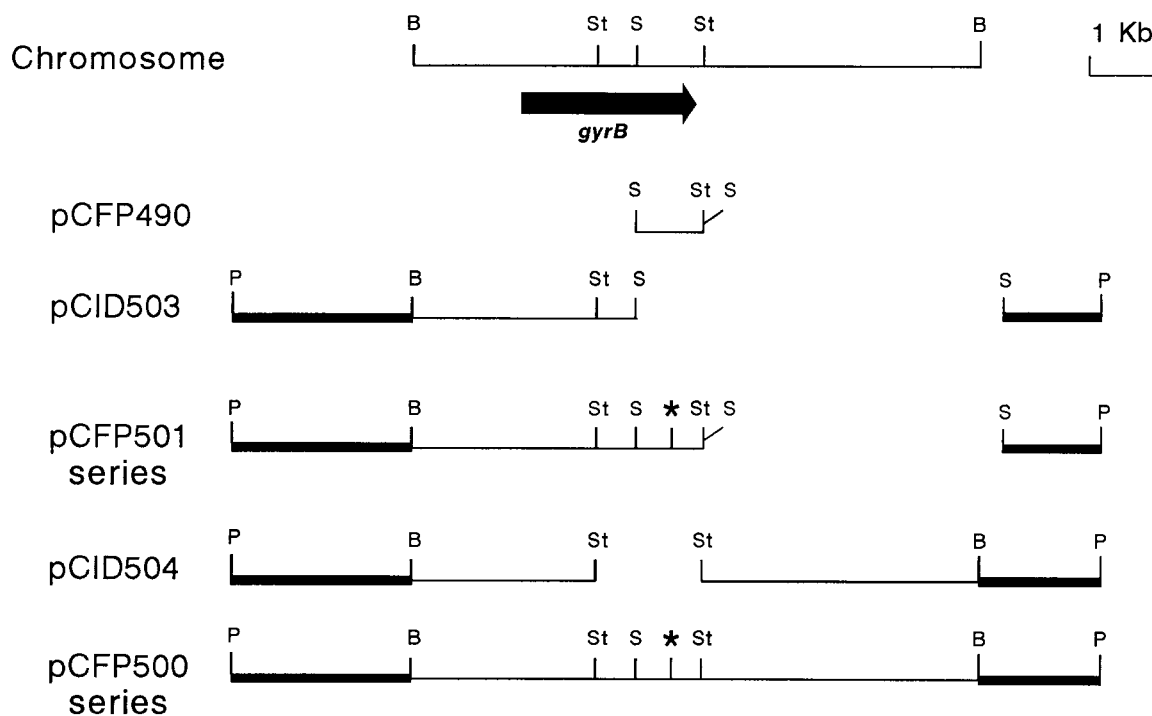


FIG. 1. Plasmids used for construction of *gyrB* mutants. Thick lines indicate sequences from pBR322, and thin lines indicate chromosomal sequences. Stars represent the engineered point mutations. B, *Bam*HI; P, *Pst*I; S, *Sal*I; St, *Stu*I.

polypeptides. Strain LE316 (18), which carries a *gyrB*(Ts) mutant gene on its chromosome, was transformed with the pCFP501 series of plasmids. Transformants harboring plasmids that carried *gyrB* W751 substitutions E, F, G, H, and K grew at the restrictive temperature (42°C), so we concluded that these mutations do not abolish GyrB function. In addition, all transformants carrying plasmids with *gyrB* W751 substitutions displayed higher resistance to MccB17 than strain LE316, harboring a wild-type *gyrB* plasmid, when tested by the cross-streaking method (22) at 42°C.

On the other hand, transformants harboring plasmid pCFP501ΔW did not grow at 42°C. To discern whether the effect of the ΔW751 mutation on GyrB function was temperature dependent [i.e., whether the mutation generated a GyrB(Ts) polypeptide unable to complement the *gyrB*(Ts) mutation of strain LE316], we performed the following experiment. All derivatives of plasmid pCID503 (including pCFP501ΔW) carry the *gyrB320* mutation (7), which confers resistance to coumermycin A<sub>1</sub>, a coumarin antibiotic. In contrast, strain LE316 is sensitive to coumermycin A<sub>1</sub> at 30°C (7). LE316 harboring pCFP501ΔW did not grow at 30°C in the presence of coumermycin A<sub>1</sub> (16 μg ml<sup>-1</sup>), while LE316 harboring other plasmids from the pCFP501 series (also carrying *gyrB320*) did grow. This result indicated that the in-frame deletion of codon 751 abolishes GyrB function irrespective of temperature.

**Construction of chromosomal *gyrB* mutants.** We intended to replace the mutant alleles for the chromosomal wild-type allele by homologous recombination. To obtain a longer homology region downstream of the mutation point, we subcloned the 1.5-kb *Stu*I fragment from every pCFP501 series

plasmid into the *Stu*I site of plasmid pCID504, creating recombinant plasmids pCFP500E, pCFP500F, pCFP500G, pCFP500H, and pCFP500K. Plasmid pCID504 (7) carries the same *gyrB320* (Cou<sup>r</sup>) mutation harbored by pCID503 (Fig. 1).

Allelic replacement was carried out by transforming a *recBC sbcBC* strain with the linearized plasmids of the pCFP500 series (26) and selecting for resistance to coumermycin A<sub>1</sub>. To determine which Cou<sup>r</sup> clones harbored the desired *gyrB* W751 mutations, chromosomal DNA from each Cou<sup>r</sup> clone was used as a template for allele-specific PCR amplifications of a 472-bp intragenic *gyrB* fragment. Finally, all *gyrB* W751 mutations were P1 transduced to strain RYC1010 (RYC1000 λp *recA cIind*) (23) for tests of MccB17 sensitivity and DNA gyrase function. The selection procedure that we used was identical to the one outlined above (resistance to coumermycin A<sub>1</sub> plus allele-specific PCR). The resultant strains carried the desired substitutions: W751E (strain CFP1020E), W751F (CFP1020F), W751G (CFP1020G), W751H (CFP1020H), and W751K (CFP1020K).

**Phenotypic characterization of *gyrB* mutants.** We assayed the effect of MccB17 on the viability of the *gyrB* mutants (Fig. 2). As controls, we used the isogenic wild-type strain RYC1020 (RYC1010 *gyrB320*) and the MccB17<sup>r</sup> mutant isolated by in vivo methods [RYC1030; relevant genotype, RYC1020 *gyrB301* (W751R)]. Strains were grown in 20 ml of M63 glucose minimal medium to an optical density at 600 nm of 1.5. Cells were then collected by centrifugation and resuspended in 4 ml of fresh M63 medium. Aliquots of cells were treated with different doses of MccB17 at 37°C for 1 h, plated out on Luria-Bertani (LB) medium, and incubated at 37°C to deter-

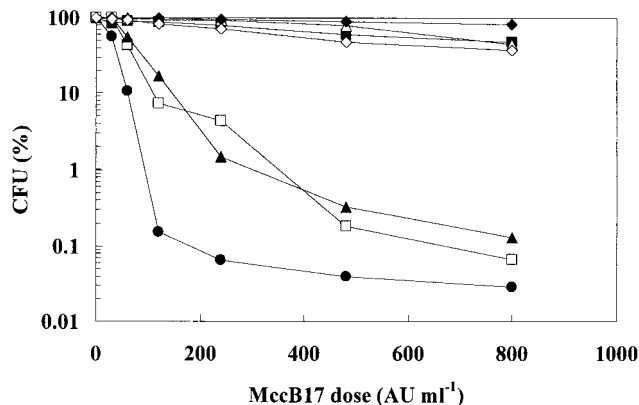


FIG. 2. Effect of MccB17 on the viability of *E. coli gyrB* mutant strains. Aliquots of cells were treated with different doses of MccB17 at 37°C for 1 h, plated out on LB medium, and incubated at 37°C to determine the number of CFU. Values are the means of three independent experiments. Symbols: ●, wild-type RYC1020; ■, CFP1020E W751E mutant; □, CFP1020F W751F mutant; △, CFP1020G W751G mutant; ▲, CFP1020H W751H mutant; ◆, CFP1020K W751K mutant; ◇, RYC1030 W751R mutant. AU, antibiotic units; CFU (%), percentage of CFU surviving after MccB17 treatment.

mine the number of CFU. MccB17 stocks were obtained and titrated as described before (14).

While all mutant strains were less sensitive to MccB17 than the wild-type strain, the higher levels of resistance were due to mutations which replaced large hydrophilic polar side chains (lysine, glutamic acid, and arginine) or very small side chains (glycine) with the hydrophobic side chain of tryptophan (Fig. 2). Interestingly, mutants that harbored aromatic-for-aromatic substitutions of residue 751, such as phenylalanine (nonpolar) or histidine (polar) for tryptophan, retained a significant level of MccB17 sensitivity (Fig. 2). Taken together, these results suggest that removal of the aromatic side chain of tryptophan plays a major role in bringing about MccB17 resistance, while increasing the polarity of the side chain of residue 751 is not sufficient by itself to prevent the inhibition of DNA gyrase by MccB17.

We next tested the effect of *gyrB* mutations on growth rate. No differences in growth rate were observed in LB rich medium. However, strains CFP1020E and CFP1020K grew more slowly in M63 glucose minimal medium than the wild-type strain (data not shown). This result suggested that DNA gyrase activity might be altered in these mutants.

To explore this possibility, we assayed the effect of *gyrB* W751 mutations on the supercoiling of a reporter plasmid (Fig. 3). Wild-type strain RYC1020 (W) and its *gyrB* mutant derivatives (E, F, G, H, K, and R) were transformed with multicopy plasmid pPH16 (6). Strains were grown in LB medium supplemented with 40  $\mu$ g of ampicillin per ml, and plasmid DNA was isolated by using Plasmid DNA Midi kits (Qiagen). Plasmid topoisomers were separated in 0.8% agarose-chloroquine gels as described (15). Plasmids extracted from strains CFP1020E and CFP1020K showed higher levels of negative supercoiling than the plasmid extracted from the control strain (Fig. 3, lanes W, E, and K), which might account for the slower growth rate of these two mutant strains, since it is known that excess neg-

ative supercoiling impairs many cellular processes (24). Most of the plasmids isolated from strains CFP1020G and CFP1020H appeared as catenanes (Fig. 3, lanes G and H). This result indicated that separation of daughter molecules after plasmid replication, a process dependent on the decatenating activity of DNA gyrase, was taking place in those mutants at an unusually slow rate. Finally, plasmids extracted from strains CFP1020F and RYC1030 (W751R mutant) showed a supercoiling level roughly similar to that of the plasmid extracted from the wild-type strain (Fig. 3, lanes W, F, and R).

Taken together, our results highlight the role of the tryptophan 751 residue of the GyrB polypeptide in the biological activity of DNA gyrase. In fact, the alignment of sequences of type II DNA topoisomerases from different organisms reveals extensive similarities in the sequence stretch that includes this residue (Fig. 4). In bacteria and archaea, residues at the position equivalent to *E. coli* GyrB W751 are tryptophan or arginine. Remarkably, the MccB17-resistant mutants isolated by in vivo methods carry the W751R substitution, which in our experiments showed a normal growth rate with a supercoiling level similar to that of the wild type. Other substitutions generated by in vitro mutagenesis resulted in either altered DNA gyrase activity (high supercoiling levels concomitant with a diminished growth rate [W751E and W751K] or a deficiency in decatenating activity [W751G and W751H]) or low-level resistance to MccB17 (W751F).

Although the crystal structure of the *E. coli* GyrB N-terminal domain has already been solved (25), no data are available for the C-terminal domain. However, the crystal structure of a large fragment of yeast type II topoisomerase containing the domain homologous to the GyrB C terminus (the so-called B' domain) has been solved at a resolution of 2.7 Å (3). The position equivalent to *E. coli* GyrB W751 is occupied by arginine 615 in the yeast enzyme (Fig. 4), a residue that is located

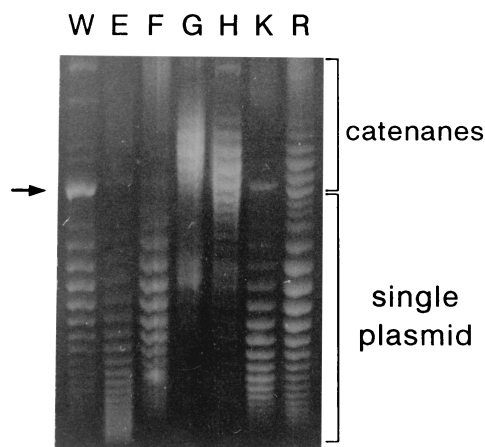


FIG. 3. Effect of *gyrB* mutations on the supercoiling of a reporter plasmid. Plasmid pPH16 topoisomers isolated from wild-type strain RYC1020 (W) and its *gyrB* mutant derivatives (E, F, G, H, K, and R) were separated in 0.8% agarose-chloroquine gels. Electrophoresis was performed in 90 mM Tris-borate (pH 8.3)-10 mM EDTA-10  $\mu$ g of chloroquine per ml at 3 V  $\text{cm}^{-1}$  for 18 h. Under these conditions, the more negatively supercoiled topoisomers migrate faster. The arrow indicates the position of pPH16 DNA after in vitro treatment with DNA topoisomerase I to achieve complete relaxation.

GyrB subunits of DNA gyrase		
<i>Escherichia coli</i>	735	SIQRYKGLGEMNPEQLWETTMDPESRRML 763
<i>E. coli</i> MccB17 <sup>R</sup> mutant	735	SIQRYKGLGEMNPEQLRETMDPESRRML 763
<i>Haemophilus influenzae</i>	739	EVQRYKGLGEMNADQLWETTMDPNSRRML 777
<i>Bacillus subtilis</i>	570	GLQRYKGLGEMNATQLWETTMDPSSRTLL 598
<i>Streptococcus pneumoniae</i>	581	TIQRYKGLGEMDDHQLWETTMDEPHRLMA 609
<i>Haloferax sp</i>	571	QVQRFKGLGEMNPDQLWDTMNPENRVLK 599
<i>Mycoplasma pneumoniae</i>	582	NLQRYKGLGEMDAIQLWETTMDEPKVRTLL 610
<i>Mycobacterium tuberculosis</i>	618	GIQRYKGLGEMDAKELWETTMDESVRVLR 646
<i>Streptomyces coelicolor</i>	618	SVQRFKGLGEMNAEELRITTMDEHRVLR 646
		* * * * *
ParE subunits of Topoisomerase IV		
<i>Escherichia coli</i>	560	NVQRFKGLGEMNPMQLRETTLDPNTRRLV 588
<i>Haemophilus influenzae</i>	560	NVQRFKGLGEMNPSQLRETTMDPNTRRLV 588
<i>Bacillus subtilis</i>	573	TIQRYKGLGEMNADQLWETTMNPESRTLV 601
<i>Streptococcus pneumoniae</i>	576	TLQRYKGLGEMNADQLWETTMNPETRTLI 605
<i>Mycoplasma pneumoniae</i>	569	NVQRYKGLGEMNADQLWETTMNPMTRKLI 597
		* * * * *
<i>Escherichia coli</i> GyrB	735	SIQRYKGLGEMNPEQLWETTMDPESRRML 763
<i>E. coli</i> GyrB MccB17 <sup>R</sup> mutant	735	SIQRYKGLGEMNPEQLRETMDPESRRML 763
<i>Saccharomyces cerevisiae</i> Topo II	599	KQRYKGLGTSLAQEVREYFNSLDRHLKI 627
		* * * * *

FIG. 4. Alignment of amino acid sequences of type II DNA topoisomerases from different organisms. Prokaryotic type II topoisomerases include GyrB polypeptides from DNA gyrase and ParE polypeptides from DNA topoisomerase IV. Stars indicate identical amino acids. The residues equivalent to *E. coli* GyrB tryptophan 751 appear in bold.

in the B' fragment, as part of the  $\alpha 8$  helix. It should be noted that in most prokaryotic type II topoisomerases,  $\alpha 8$  helix would be either kinked or truncated six residues downstream of W751 due to the existence of a proline residue in the position equivalent to *Saccharomyces cerevisiae* leucine 621 (Fig. 4). The results of recent studies on the mechanisms of type II DNA topoisomerases support a model in which the enzyme acts as an ATP-dependent molecular clamp with two gates for the strand passage (20, 21). In this model, the DNA segment to be transported (T-segment) enters through the gate on one side of the enzyme, crosses the cleaved, enzyme-bound DNA segment (G-segment), and, moving between the two monomers of the enzyme, exits through a second gate on the opposite side of the complex (3, 10, 20, 21). Interestingly, the B' domain is part of the entry gate, and so it is tempting to speculate on a putative direct interaction of the hydrophobic MccB17 with GyrB W751 and residues in its spatial vicinity, an interaction that may block strand passage.

MccB17 is the only peptide inhibitor of DNA gyrase whose resistance mutations map within the B' domain of GyrB. Mutations conferring resistance to MccB17 have been shown to exert very diverse effects on in vivo DNA gyrase activity. Therefore, MccB17 should be a valuable tool for probing the role of the B' domain in the DNA gyrase protein complex.

F. J. del Castillo was the recipient of a fellowship from Fundación Ramón Areces.

#### REFERENCES

- Adachi, T., M. Mizuuchi, E. A. Robinson, E. Appella, M. H. O'Dea, M. Gellert, and K. Mizuuchi. 1987 DNA sequence of the *E. coli* *gyrB* gene: application of a new sequencing strategy. *Nucleic Acids Res.* **15**:771-784.
- Baquero, F., and F. Moreno. 1984. The microcins. *FEMS Microbiol. Lett.* **23**:117-124.
- Berger, J. M., S. J. Gamblin, S. C. Harrison, and J. C. Wang. 1996. Structure and mechanism of DNA topoisomerase II. *Nature* **379**:225-232.
- Brown, P. O., C. L. Peebles, and N. R. Cozzarelli. 1979. A topoisomerase from *Escherichia coli* related to DNA gyrase. *Proc. Natl. Acad. Sci. USA* **76**:6110-6114.
- Davagnino, J., M. Herrero, D. Furlong, F. Moreno, and R. Kolter. 1986. The DNA replication inhibitor microcin B17 is a forty-three-amino-acid protein containing sixty percent glycine. *Proteins* **1**:230-238.
- del Castillo, F. J., S. C. Leal, F. Moreno, and I. del Castillo. 1997. The *Escherichia coli* K-12 *sheA* gene encodes a 34-kDa secreted haemolysin. *Mol. Microbiol.* **25**:107-115.
- del Castillo, I., J. L. Vizán, M. C. Rodríguez-Sáinz, and F. Moreno. 1991. An unusual mechanism for resistance to the antibiotic coumermycin A<sub>1</sub>. *Proc. Natl. Acad. Sci. USA* **88**:8860-8864.
- Drlica, K. 1984. Biology of bacterial deoxyribonucleic acid topoisomerases. *Microbiol. Rev.* **48**:273-289.
- Drlica, K., and R. J. Franco. 1988. Inhibitors of DNA topoisomerases. *Biochemistry* **27**:2253-2259.
- Fass, D., C. E. Bogden, and J. M. Berger. 1999. Quaternary changes in topoisomerase II may direct orthogonal movement of two DNA strands. *Nat. Struct. Biol.* **6**:322-326.
- 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. USA* **73**:3872-3875.
- Gellert, M., L. M. Fisher, and M. H. O'Dea. 1979. DNA gyrase: purification and properties of a fragment of the gyrase B protein. *Proc. Natl. Acad. Sci. USA* **76**:6289-6293.
- Genilloud, O., F. Moreno, and R. Kolter. 1989. DNA sequence, products, and transcriptional pattern of the genes involved in production of the DNA replication inhibitor microcin B17. *J. Bacteriol.* **171**:1126-1135.
- Herrero, M., and F. Moreno. 1986. Microcin B17 blocks DNA replication and induces the SOS system in *Escherichia coli*. *J. Gen. Microbiol.* **132**:393-402.
- Higgins, C. F., C. J. Dorman, D. A. Stirling, L. Waddell, I. R. Booth, G. May, and E. Bremer. 1988. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. *Cell* **52**:569-584.
- Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367-382.
- Maxwell, A. 1997. DNA gyrase as a drug target. *Trends Microbiol.* **5**:102-109.
- 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* K12. *Mol. Gen. Genet.* **177**:103-112.
- Reece, R. J., and A. Maxwell. 1991. DNA gyrase: structure and function. *Crit. Rev. Biochem. Mol. Biol.* **26**:335-375.
- Roca, J., and J. C. Wang. 1994. DNA transport by a type II DNA topoisomerase: evidence in favor of a two-gate mechanism. *Cell* **77**:609-616.
- Roca, J., J. M. Berger, S. C. Harrison, and J. C. Wang. 1996. DNA transport by a type II topoisomerase: direct evidence for a two-gate mechanism. *Proc. Natl. Acad. Sci. USA* **93**:4057-4062.
- San Millán, J. L., C. Hernández-Chico, P. Pereda, and F. Moreno. 1985. Cloning and mapping of the genetic determinants for microcin B17 production and immunity. *J. Bacteriol.* **163**:275-281.
- Vizán, J. L., C. Hernández-Chico, I. del Castillo, and F. Moreno. 1991. The peptide antibiotic microcin B17 induces double-strand cleavage of DNA mediated by *E. coli* DNA gyrase. *EMBO J.* **10**:467-476.
- Wang, J. C. 1996. DNA topoisomerases. *Annu. Rev. Biochem.* **65**:635-692.
- Wigley, D. B., G. J. Davies, E. J. Dodson, A. Maxwell, and G. Dodson. 1991. Crystal structure of an N-terminal fragment of the DNA gyrase B protein. *Nature* **351**:624-629.
- Winans, S. C., S. J. Elledge, J. H. Krueger, and G. C. Walker. 1985. Site-directed insertion and deletion mutagenesis with cloned fragments in *Escherichia coli*. *J. Bacteriol.* **161**:1219-1221.
- Yorgey, P., J. Lee, J. Kördel, E. Vivas, P. Warner, D. Jebaratnam, and R. Kolter. 1994. Posttranslational modifications in microcin B17 define an additional class of DNA gyrase inhibitor. *Proc. Natl. Acad. Sci. USA* **91**:4519-4523.