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DNA Gyrase from the Albicidin Producer *Xanthomonas albilineans* Has Multiple-Antibiotic-Resistance and Unusual Enzymatic Properties▽

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The sugarcane pathogen *Xanthomonas albilineans* produces a family of antibiotics and phytotoxins termed albicidins, which inhibit plant and bacterial DNA gyrase supercoiling activity, with a 50% inhibitory concentration (50 nM) comparable to those of coumarins and quinolones. Here we show that *X. albilineans* has an unusual, antibiotic-resistant DNA gyrase. The *X. albilineans gyrA* and *gyrB* genes are not clustered with previously described albicidin biosynthesis and self-protection genes. The GyrA and GyrB products differ from *Escherichia coli* homologues through several insertions and through changes in several amino acid residues implicated in quinolone and coumarin resistance. Reconstituted *X. albilineans* DNA gyrase showed 20- to 25-fold-higher resistance than *E. coli* DNA gyrase to albicidin and ciprofloxacin and 8-fold-higher resistance to novobiocin in the supercoiling assay. The *X. albilineans* DNA gyrase is unusual in showing a high degree of distributive supercoiling and little DNA relaxation activity. *X. albilineans* GyrA (XaA) forms a functional gyrase heterotetramer with *E. coli* GyrB (EcB) and can account for albicidin and quinolone resistance and low levels of relaxation activity. XaB probably contributes to both coumarin resistance and the distributive supercoiling pattern. Although XaB shows fewer apparent changes relative to EcB, the EcA · XaB hybrid relaxed DNA in the presence or absence of ATP and was unable to supercoil. A fuller understanding of structural differences between albicidin-sensitive and -resistant gyrases may provide new clues into features of the enzyme amenable to interference by novel antibiotics.

DNA topoisomerases perform essential roles during the processes of DNA transcription, replication, and recombination (1, 10, 40). Bacterial DNA gyrase has the unique ability to introduce negative supercoils into DNA, an activity essential to removal of excess positive supercoils generated when complementary strands are separated during replication of a circular chromosome and to maintain a homeostatic level of negative supercoiling to facilitate processes such as transcription. After wrapping around the gyrase A_2B_2 protein complex, a DNA loop is passed through a transient break in the bound DNA backbone, which is then resealed, and the DNA is released from the enzyme. This intramolecular strand passage results in the introduction of two negative supercoils (10, 13). The GyrA subunit is required for the bending, cleavage, and reunion of DNA, and the GyrB subunit is required for the transduction of energy from ATP binding and hydrolysis into the mechanism for DNA strand passage. In vitro, the enzyme can work at lower efficiency to relax supercoiled DNA without energy from ATP.

Bacterial DNA topoisomerase IV in vivo is believed to perform intermolecular strand passage (decatenation) for the partitioning of replicated bacterial chromosomes (22) and to relax excessive supercoiling from DNA gyrase action (43). Composed of subunits encoded by *parC* and *parE*, which are homologous to *gyrA* and *gyrB*, it preferentially relaxes positive supercoils and lacks supercoiling activity attributed to structural differences between the GyrA and ParC C-terminal DNA-binding domains (13, 19, 34).

Bacterial DNA gyrase differs substantially from eukaryotic topoisomerases and is the target of several classes of antibiotics (26). The quinolones, including nalidixic acid and oxolinic acid, and the peptide antibiotics microcin B17 and CcdB act by interfering with the DNA-rejoining step. In contrast, the coumarins, including coumermycin A_1 and novobiocin, compete with ATP for binding to the GyrB subunit (26). Simocyclinones inhibit an early step of the gyrase catalytic cycle by preventing binding of the enzyme to DNA (15).

Albicidin antibiotics and phytotoxins from the sugarcane pathogen *Xanthomonas albilineans* inhibit DNA replication in bacteria and in sugarcane proplastids (4, 5). Albicidins cause the characteristic chlorotic symptoms of sugarcane leaf scald disease by blocking chloroplast development, and they play additional roles in systemic disease establishment (2, 46). Albicidins are potent inhibitors of the supercoiling activity of bacterial and plant DNA gyrases, with a 50% inhibitory concentration (IC_{50}) (40 to 50 nM) comparable to those of coumarins and quinolones but with some unique mechanistic effects (17). This prompted us to test whether *X. albilineans* has an albicidin-resistant DNA gyrase in addition to previously identified self-protection mechanisms of regulated biosynthesis (44), efficient export (9), and a DNA-mimicking protein (17).

This paper describes the cloning and characterization of *gyrA* and *gyrB* from *X. albilineans* and purification of the corresponding enzyme subunits. The reconstituted enzyme has unusual functional properties, including multiple antibiotic resistance, distributive supercoiling, and little relaxation activity in vitro. By analysis of sequence features and hybrid enzymes,

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TABLE 1. Properties of strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
E. coli		
DH5a	Φ80d*lacZ*Δ*M15*, Δ(*lacZYA–argF*)*U169*	Promega
BL21	(λDE3)pLysS	Invitrogen
KNK546	F^- *polA thyA uvrA phx gyrA43* (Ts)	24
N4177	*strA galK gyrB221* (Cou^r) *gyrB203* (Ts); Cou^r mutation (Arg136Cys); Ts mutation (Pro171Ser)	27
TOP10	F^- Δ(*mrr–hsdRMS–mcrBC*) Δ(*are–leu*)*7697* Δ*lacX74*	Invitrogen
X. albilineans		
Xa13	Wild-type albicidin producer from sugarcane (Queensland, Australia); Ap^r	44
LS135	Albicidin-leaky mutant of LS155	39
LS155	Wild-type albicidin producer from sugarcane (Queensland, Australia); Ap^r	39
Plasmids		
pBluescript II	Expression vector; native protein; *bla* (Ap^r)	Stratagene
pET-19b	Expression vector; His_{10} fusion protein (N terminal); *bla* (Ap^r)	Invitrogen
pET-GyrB19b	pET-19b containing *gyrB*; Ap^r	This study
pET-29b	Expression vector; His_6 fusion protein (C terminal); *bla* (Km^r)	Invitrogen
pET-GyrA29b	pET-29b containing *gyrA*; Ap^r	This study
pBR322	Substrate DNA for DNA gyrase assays	8

[a] Ts, temperature sensitive; Cou, coumermycin; Ap, ampicillin; Km, kanamycin.

including *X. albilineans* and *Escherichia coli* subunits, we have identified structural regions likely to contribute to these unusual functional properties of the *X. albilineans* DNA gyrase.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Properties of bacteria and plasmids used in this study are listed in Table 1. *X. albilineans* strains were routinely cultured on sucrose peptone medium at 28°C (3). *E. coli* strains DH5α and Top10 used as hosts in cloning experiments were grown on LB medium at 37°C (35). Broth cultures were aerated by shaking at 200 rpm on an orbital shaker. Growth of liquid cultures was measured by determining the optical density at 600 nm. The following antibiotics were added to media as required: kanamycin (50 μg/ml); ampicillin (100 μg/ml); and chloramphenicol (30 μg/ml).

Routine genetic procedures. DNA isolation, restriction digestions, ligation reactions, and transformation and electrophoresis of DNA and proteins were performed as described by Sambrook et al. (35). High-fidelity *Taq* polymerase (Invitrogen) with proofreading activity was used in all PCRs. DNA fragments were excised from agarose gels, and residual agarose was removed using a BresaClean DNA purification kit (GeneWorks, Adelaide, Australia).

Isolation and sequencing of *gyrA* and *gyrB*. Primers XalbgyrAfwd (5′-ATG GCA GAA ACC GCC AAG GAA ATC-3′) and XalbgyrArev1 (5′-TTG CCG TTG CGC TCG GTG G-3′) were designed based on the nucleotide sequences of *gyrA* from *Xanthomonas campestris* (GenBank accession no. NP_636945.1) and *Xanthomonas axonopodis* (NP_641963.1). Amplification of *X. albilineans gyrA* was achieved with an initial denaturation at 94°C for 10 min followed by five cycles of denaturation for 1 min at 94°C, 1 min of annealing at 50°C, and 2 min of polymerase extension at 72°C, 25 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C, and then a final extension for 10 min at 72°C followed by cooling to 4°C. The sequences for the 5′ and 3′ ends of the gene were obtained by sequencing directly from the genomic DNA of *X. albilineans* by use of primers corresponding to the regions inside the primers listed above. The *X. albilineans gyrB* gene was initially isolated and sequenced during investigation of sequences flanking the Tn*5* insertion in leaky Tox-negative mutant LS135 (39).

Sequencing reactions were performed by dideoxynucleotide chain termination (36) using a BigDye Terminator cycle sequencing kit and 373A DNA sequencer (PE Applied Biosystems) available from the Australian Genome Research Facility. Oligonucleotide primers were purchased from GeneWorks (Adelaide, Australia). DNA and protein sequences were compared in the GenBank, EMBL, and Swiss-Prot databases (accession date, 3 Dec 2006). Molecular weights and extinction coefficients for GyrA and GyrB were calculated using Vector NTi version 9 (InforMax).

Construction of expression vectors for GyrA and GyrB. The coding regions of *gyrA* and *gyrB* were amplified using PCR from genomic DNA of *X. albilineans* strain LS155. Primer GyrA_fwd_2 (5′-GAA CCC ATA TGG CAG AAA CCG CCA AGG-3′) contained an NdeI restriction site (underlined), whereas primer GyrA_rev_XhoI (5′-ATC AGC CCT CGA GCG ACG CGG TG-3′) introduced an XhoI site (underlined) overlapping the stop codon. The amplified *gyrA* was digested using NdeI/XhoI and ligated into pET-29b to give pET-GyrA29b, which resulted in a six-His tag at the carboxy terminal.

The coding region of *gyrB* was amplified using primers XagyrB_fwd (5′-CCT ACT GCG AGC CAT ATG ACC GAC GAA CAG-3′) and XagyrB_rev (5′-AGA CAT GGC AAG GGA TCC GTC CCG GCA TAG-3′) containing restriction sites (underlined) and cloned into NdeI/BamHI sites of pET-19b to yield pET-GyrB19b, introducing a deca-His tag at the amino terminal.

Purification of GyrA and GyrB subunits. *E. coli* BL21(λDE3)pLysS carrying pET-GyrA29b or pET-GyrB19b was grown at 37°C overnight in 20 ml of LB medium containing 30 μg/ml of chloramphenicol and 100 μg/ml of ampicillin for GyrB or 50 μg/ml of kanamycin for GyrA. Bacteria were harvested by centrifugation at 2,000 × *g* for 10 min at room temperature. The pellet was suspended in 20 ml of LB and used to inoculate 150 ml of LB containing the selective antibiotics. Cells were grown at 30°C until the optical density at 600 nm reached 0.4 for GyrA and 0.6 for GyrB. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to reach a final concentration of 0.5 mM, and growth was continued for a further 3 h. Bacterial cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C, and the pellet was suspended in 12 ml of binding buffer (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, and 5 mM imidazole). The cells were snap frozen in liquid N_2 and stored at −80°C until further use.

The suspension was thawed on ice, lysozyme was added to reach a final concentration of 0.1%, and the mixture was incubated on ice for 30 min. The cells were then disrupted by sonication on ice using a Branson 250 sonifier (using four 20-s bursts with a 30-s rest between cycles and a Microtip probe set to 50-w power output) and centrifuged at 45,000 × *g* for 20 min at 4°C. The supernatant was mixed with 4 ml of 50% nickel-nitrilotriacetic acid resin (Qiagen) in a sterile precooled tube and gently agitated at 4°C for 1 h. The suspension was centrifuged at 700 × *g* for 2 min at 4°C and washed with 30 ml of binding buffer and then four times with 30 ml of wash buffer (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, and 60 mM imidazole). The washed resin was then resuspended in 2 ml of elution buffer (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, and 250 mM imidazole), and the His-tagged proteins were eluted through a filtration column. The eluted proteins were dialyzed at 4°C overnight in 2.5 liters of 50 mM Tris-HCl (pH 7.9), 30% glycerol, 1 mM dithiothreitol (DTT),and 1 mM EDTA. The dialyzed proteins were flash frozen in liquid N_2 and stored at −80°C. Protein purity was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Protein concentrations were quantified using a spectrophotometer (Nanodrop ND-1000) at 280 nm.

DNA gyrase assays. Supercoiling activity was tested using various ratios of purified *X. albilineans* DNA gyrase A and B subunits. *E. coli* DNA gyrase (Inspiralis Ltd., Norwich, United Kingdom) was used as a positive control.

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FIG. 1. Comparison of the amino acid sequences of the DNA gyrase A subunits from *E. coli* (Ec; GenBank accession no. NP_754659.1), *X. albilineans* (Xa; this study), *X. axonopodis* (Xax; NP_636945.1), and *X. campestris* (Xc; NP_636945.1). Identical amino acids are represented by

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Compatibility of *X. albilineans* and *E. coli* DNA gyrase subunits was tested at various ratios. The methanol concentration in reactions testing albicidin sensitivity was constant at 1.25% (vol/vol).

Gyrase supercoiling reaction mixtures (30 μl) containing gyrase (1 U), buffer (35 mM Tris-HCl [pH 7.5], 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM spermidine, 1 mM ATP, 6.5% [wt/vol] glycerol, 0.1 mg/ml bovine serum albumin), and 5 nM (0.4 μg) of relaxed pBR322 DNA were incubated at 37°C for 30 min and then terminated by addition of a 0.5 volume of STEB (20% [wt/vol] sucrose, 0.05 M Tris-HCl [pH 7.5], 0.05 M EDTA, 50 μg/ml bromophenol blue) and 2 volumes of chloroform-isoamyl alcohol (24:1) followed by electrophoretic analysis in 1% agarose gels. Gyrase relaxation assays were performed similarly to the supercoiling assays except that ATP and spermidine were omitted and the substrate was supercoiled pBR322 DNA. Gels were viewed and photographed on a GelDoc system (Bio-Rad) using autoexposure. Intensities of bands were calculated using one-dimensional Totallab TL100 gel analysis software (version 2006b; Nonlinear Dynamics). GraphPad Prism (version 4.0) was used to analyze the data and determine IC_{50} values using a one-phase exponential decay curve.

DNA cleavage reactions contained gyrase (2 U), 5 to 12 nM relaxed pBR322 DNA, 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM spermidine, 0.1 mg/ml bovine serum albumin, 6.5% (wt/vol) glycerol, and various concentrations of drug in the presence of 1 mM ATP. Linear product was released by the addition of SDS to 0.2% (wt/vol) and proteinase K to 0.1 mg/ml followed by incubation at 37°C for 30 min. Samples were analyzed by electrophoresis in 1% agarose (in the presence of 1 μg/ml of ethidium bromide).

Preparation of albicidin and assay of albicidin resistance. Albicidin was prepared from *X. albilineans* Xa13 and quantified as described previously (3). The HPLC-purified α peak of albicidin was used in all assays. Albicidin resistance was quantified by a microbial plate bioassay as described previously (45).

Nucleotide sequence accession numbers. The full-length sequences of *X. albilineans gyrA* and *gyrB* obtained in this study have been deposited in GenBank under accession no. EU267170 and EU267171, respectively.

RESULTS

Cloning and sequencing of the *gyrA* and *gyrB* genes from *X. albilineans*. Attempts to clone the full-length *X. albilineans gyrA* in pBlueScript II were unsuccessful. DNA supercoiling in *E. coli* is controlled by a homeostatic mechanism (27), and overexpression of the introduced *gyrA* probably inhibited growth through a compositional imbalance of gyrase subunits or excessive DNA supercoiling. To overcome this, *gyrA* was cloned behind the T7 promoter in pET-19b (only induced in strains carrying the T7 polymerase gene).

The full-length *gyrB* gene was contained along with a Tn*5* insertion in a 2.7-kb EcoRI fragment cloned from *X. albilineans* Tox-negative leaky mutant LS135 into pBluescript II.

Bioinformatic analysis of *gyrA* and *gyrB*. Analysis of the cloned *gyrA* sequence revealed an open reading frame of 2,805 bp encoding a 935-amino-acid protein with an M_r of 102,840 (Fig. 1). The deduced amino acid sequence showed highest (83%) identity to that of the DNA GyrA subunit of *X. campestris* (GenBank accession no. NP_636945.1) and *X. axonopodis* (NP_641963.1). It showed 62% identity to that of the *E. coli* DNA GyrA subunit (NP_754659.1) (Fig. 1).

A FASTA search of the Swiss-Prot database was performed to select the 21 most similar proteins (GyrA and ParC) for construction of a protein tree revealing GyrA and ParC groups (data not shown). The identities between *X. albilineans* GyrA and other GyrA proteins ranged from 58% to 83%, while identities with ParC were below 35%. As expected, the *X. albilineans* GyrA clustered closely with *X. campestris*, *X. axonopodis*, and *X. oryzae* GyrA proteins.

The *X. albilineans gyrB* open reading frame encodes a protein of 820 amino acids, with an M_r of 90,230 (Fig. 2) and 60 to 61% identity to the GyrB subunits from *E. coli* (GenBank accession no. G41646), *Salmonella enterica* serovar Typhimurium (GenBank accession no. Q60008), *Haemophilus influenzae* (GenBank accession no. G1573554), and *Pseudomonas putida* (GenBank accession no. G45694).

The promoter regions of *gyr* genes typically play a role in relaxation-stimulated transcription to regulate cellular DNA topology, and the mechanism appears to involve a propensity to curvature in the vicinity of the −10 region rather than any particular consensus sequence (38). In *X. albilineans*, sequences similar to the "extended −10" bacterial consensus promoter sequence (TGNTATAAT) (23) exist 78 bp upstream of the translational start of the *gyrB* gene and 109 bp upstream of the start of *gyrA*. Both promoters lack canonical −35 regions (TTGACA). The *E. coli gyrA* promoter similarly shows an extended −10 region without a canonical −35 region (25). In *E. coli*, the RNA polymerase sigma 70 subunit recognizes the −10 extended region, in which the TG motif plays a role in efficient initiation without a canonical −35 region (7, 23).

Purification of *X. albilineans* gyrase subunits. Expression of *X. albilineans gyrA* and *gyrB* in *E. coli* pET expression vectors and purification from 150 ml of IPTG-induced cell culture by nickel chelate chromatography yielded 1 mg and 3 mg of His-tagged proteins, respectively. SDS-polyacrylamide gel electrophoresis analysis of the gyrase subunits showed that GyrA and GyrB have the expected molecular weights of 102,000 and 90,000, respectively (data not shown).

Supercoiling activity of *X. albilineans* DNA gyrase. Combinations of GyrA and GyrB proteins were tested for DNA supercoiling activity in the presence or absence of 1 mM ATP by use of relaxed pBR322 as the substrate (Fig. 3). Gyrase subunits were reconstituted at A:B molar ratios of 80:20, 20:80, and 50:50 and were then titrated to determine the minimum for 50% supercoiling of 0.4 μg of relaxed pBR322 within 30 min in the routine assay conditions. This was designated 1 U of DNA gyrase activity. Neither subunit alone could supercoil relaxed substrate. A 20:80 ratio of the A and B subunits gave optimal supercoiling activity. All tested subunit ratios resulted in multiple DNA products of intermediate topology revealed as bands which migrate more slowly than the main, highly supercoiled product. No supercoiling was observed in the absence of ATP (Fig. 3).

Susceptibility of *X. albilineans* DNA gyrase to albicidin, ciprofloxacin, and novobiocin. In the DNA supercoiling assay, compared to the *E. coli* enzyme results, the *X. albilineans* gyrase gave IC_{50} values 25-fold higher for albicidin and ciprofloxacin and 8-fold higher for novobiocin (Fig. 4 and Table 2). In the assay for DNA cleavage induced by antibiotic in the

black characters on a gray background. Residues in *E. coli* GyrA that form the active site of the breakage-reunion reaction, including the Tyr-122 residue that covalently binds the DNA, are indicated by arrows (↓). Residues involved in quinolone resistance are indicated by filled circles (●). Residues involved in CcdB resistance in *E. coli* are indicated by filled inverted triangles (▼). Bold letters with a five-pointed star above indicate amino acids uniquely variant in *X. albilineans* from known GyrA amino acids (Swiss-Prot; accession date, 2 December 2006).

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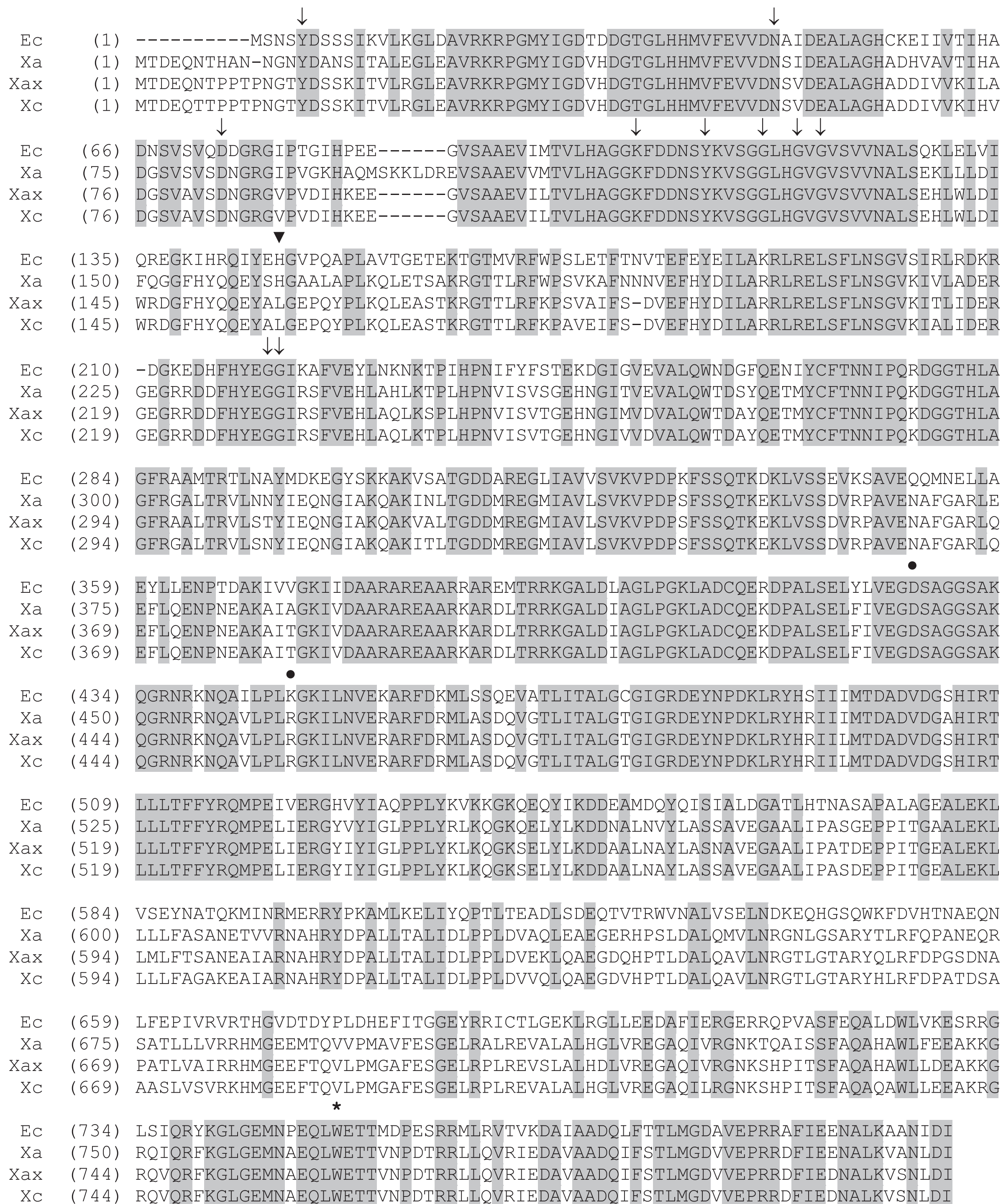

FIG. 2. Comparison of the amino acid sequences of the DNA gyrase B subunits from *E. coli* (Ec; GenBank accession no. G41646), *X. albilineans* (Xa; this study), *X. axonopodis* (Xax; NP_640360), and *X. campestris* (Xc; YP_241118). Identical amino acids are represented by black characters on a gray background. Residues important in ATP binding and hydrolysis in *E. coli* are indicated by arrows (↓). The two residues known to confer resistance to coumarin in *E. coli* are indicated by filled inverted triangles (▼). The two residues shown to be involved in quinolone resistance in *E. coli* are indicated by a filled circle (●). The residue conferring resistance to microcin A17 in *E. coli* is indicated by a five-pointed star.

presence of DNA gyrase, the *X. albilineans* enzyme gave CC_{50} values at least 4-fold higher for albicidin and at least 10-fold higher for ciprofloxacin compared to the *E. coli* enzyme values (Fig. 5). We use CC_{50} to mean the antibiotic concentration required to obtain half of the maximum yield of linear DNA observed in the reaction mixture containing antibiotic, gyrase, ATP, and DNA.

Compatibility of *X. albilineans* and *E. coli* DNA gyrase subunits. The combination of *X. albilineans* GyrA with *E. coli* GyrB formed a functional DNA gyrase (designated

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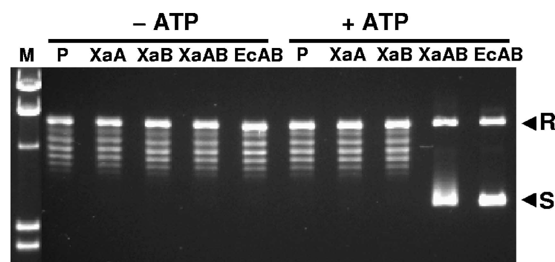

FIG. 3. Supercoiling activity of *X. albilineans* and *E. coli* DNA gyrase A and B subunits. Relaxed pBR322 (0.4 μg) was incubated with DNA GyrA of GyrB or a mixture of the two in the presence (+) or absence (−) of ATP (1 mM). Lanes: M, λDNA/HindIII; P, no DNA gyrase; XaA, *X. albilineans* DNA gyrase A; XaB, *X. albilineans* DNA gyrase B; XaAB and EcAB, reconstituted DNA gyrases (1 U each) of *X. albilineans* and *E. coli*, respectively. R and S, relaxed and supercoiled DNA, respectively.

XaA · EcB) with an IC_{50} in the supercoiling assay of 1,500, 7,500, and 100 nM for albicidin, ciprofloxacin, and novobiocin, respectively, close to the results obtained with XaAB for albicidin and ciprofloxacin and with EcAB for novobiocin (Fig. 4 and Table 2).

Unexpectedly, the *X. albilineans* DNA gyrase and the XaA · EcB hybrid enzyme were unable to relax supercoiled pBR322 even at 10 times the XaA · EcB enzyme concentration that proved effective in the supercoiling assay (Fig. 6A and B). In contrast, the EcA · XaB combination was nonfunctional for supercoiling, but it could relax supercoiled pBR322 in both the presence and the absence of ATP (Fig. 6A). This relaxation activity by the EcA · XaB enzyme was as sensitive as that of the wild-type *E. coli* DNA gyrase was to ciprofloxacin (Fig. 6C).

Combining the in vitro results with those revealing the earlier cloning effects shows that toxicity resulted from overexpression in *E. coli* of a heterologous subunit capable of reconstituting a DNA gyrase functional for supercoiling (XaA · EcB) but that overexpression of a subunit that does not form a hybrid capable of supercoiling (EcA · XaB) did not result in toxicity.

DISCUSSION

Purified, reconstituted *X. albilineans* DNA gyrase has high specific activity for supercoiling. The use of His tags facilitated purification of *X. albilineans* DNA gyrase subunits free from host (*E. coli*) proteins. The specific activity of reconstituted *X. albilineans* DNA gyrase for supercoiling was 12,500 U/mg. Different studies have reported comparable specific activities of native DNA gyrases from *E. coli* (11,500 U/mg), *Micrococcus luteus* (20,000 U/mg), *Vibrio cholerae* (28,369 U/mg), and *Bacillus subtilis* (10,000 U/mg) (32, 37, 47). Based on the optimal molar ratio of 20 A:80 B for reconstituted activity, heterologous expression of *gyrB* may yield a higher proportion of misfolded subunits. A higher specific activity of purified GyrA relative to that of GyrB has also been also reported from studies of *E. coli* (29). Native and His_6-tagged DNA gyrase subunits from *E. coli* have shown equivalent levels of activity in ATPase, DNA supercoiling, and DNA relaxation assays (16). Therefore, the His-tagged *X. albilineans* proteins were used in all subsequent assays.

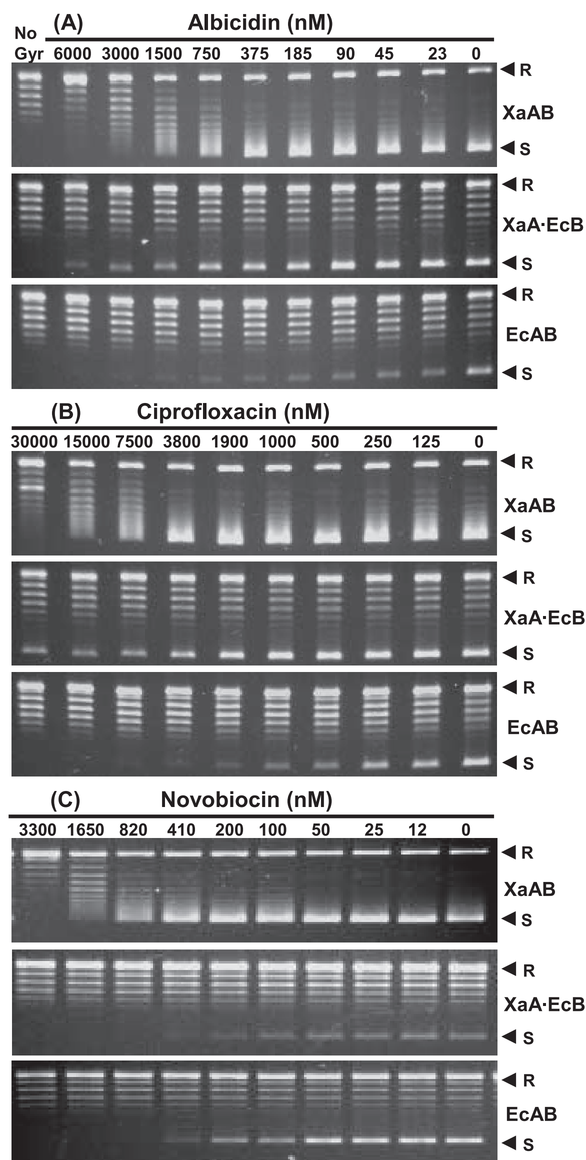

FIG. 4. Supercoiling activity of *X. albilineans* (XaAB), *E. coli* (EcAB), and hybrid (XaA · EcB) DNA gyrases in the presence of albicidin (A), ciprofloxacin (B), and novobiocin (C). Relaxed pBR322 (0.4 μg) was incubated with DNA gyrase in the presence of 1 mM ATP. R and S, relaxed and supercoiled pBR322, respectively.

***X. albilineans* DNA gyrase shows unusually distributive supercoiling.** DNA gyrases are typically highly processive, because the enzyme binds stably at the cleavage site on relaxed DNA and performs rapid supercoiling in the presence of ATP followed by rapid release of the enzyme from the supercoiled product (31). In routine supercoiling assays, this yields a single band in the supercoiled product region, as shown for the *E. coli* enzyme in Fig. 3 and Fig. 4.

In contrast, the *X. albilineans* DNA gyrase showed more distributive supercoiling, with multiple DNA bands near the main supercoiled form indicating dissociation of the enzyme from the bound DNA molecule at intermediate supercoil densities. Distributive supercoiling has been reported in studies of *E. coli* DNA gyrase variants, for example, the N-terminal 64-

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TABLE 2. Sensitivity of supercoiling activity of reconstituted DNA gyrases to antibiotics

Drug	IC_{50} (nM) for indicated reconstituted DNA gyrase(s)[a]		
	XaAB	EcAB	XaA · EcB
Albicidin	1,350	50	1,500
Ciprofloxacin	7,500	300	7,500
Novobiocin	800	100	100

[a] A, GyrA; B, GyrB; Xa, *X. albilineans*; Ec, *E. coli*.

kDa fragment of GyrA reconstituted with intact GyrB (33) and the GyrB K447E mutant which confers quinolone resistance at the expense of an 80% reduction in supercoiling specific activity (18). The mechanistic relationship between antibiotic resistance and the unusual combination of distributive supercoiling with high specific activity in the *X. albilineans* DNA gyrase remains to be determined. However, it is interesting that the XaA · EcB hybrid showed processive supercoiling (Fig. 4).

Multiple sequence changes potentially implicated in antibiotic resistance of *X. albilineans* DNA gyrase. The residues that form the active site of the breakage-reunion N-terminal domain of GyrA in *E. coli* as revealed by the crystal structure (30) are conserved in the *X. albilineans* GyrA (Fig. 1). However, the *X. albilineans* protein has two insertions, of 29 and 43 amino acids, in the C-terminal-region, which is involved in DNA wrapping and complex stability. The larger insertion is unique to the *X. albilineans* GyrA, and the shorter insertion is conserved in position but divergent in sequence in other *Xanthomonas* spp. Both are rich in negatively charged residues.

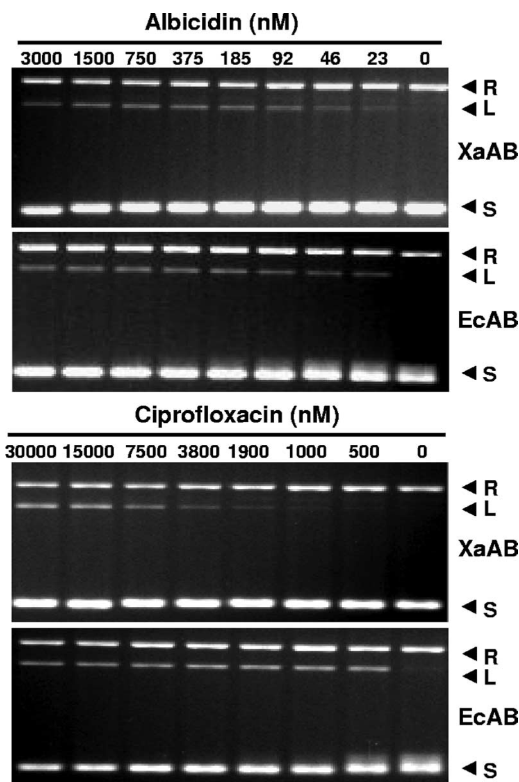

FIG. 5. Albicidin- and ciprofloxacin-induced DNA cleavage by *X. albilineans* (XaAB) and *E. coli* (EcAB) DNA gyrase. Relaxed pBR322 (0.4 μg) was used as a substrate in the presence of 1 mM ATP, 2 U DNA gyrase, and the indicated concentration of albicidin or ciprofloxacin. R and S, relaxed and supercoiled pBR322, respectively. L, linear pBR322.

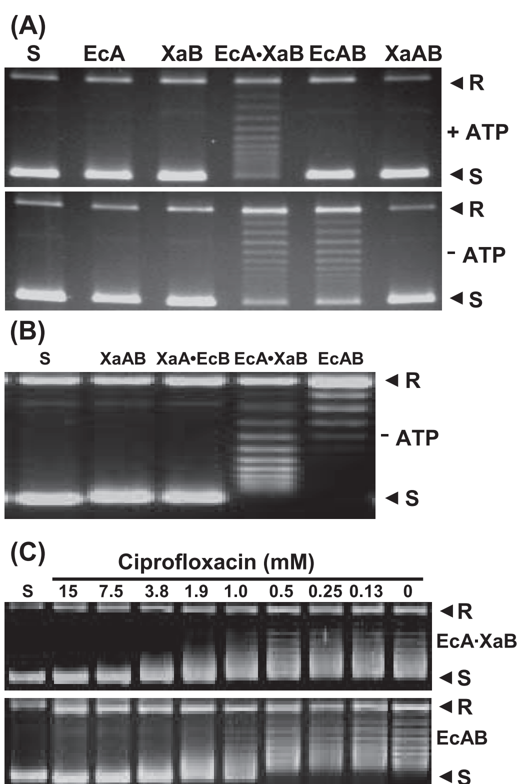

FIG. 6. Relaxation activities of *X. albilineans*, *E. coli*, or hybrid DNA gyrases. (A) Supercoiled pBR322 was incubated with DNA GyrA or GyrB or a mixture of the two in the presence (+) or absence (−) of ATP (1 mM). (B) Repeat supercoiling assay in the absence of ATP. Lanes: S, supercoiled pBR322; EcA, *E. coli* GyrA; EcB, *E. coli* GyrB; XaA · EcB, *X. albilineans* GyrB and *E. coli* GyrA; EcA · XaB, *E. coli* GyrA and *X. albilineans* GyrB; Ec AB, *E. coli* GyrA and GyrB; XaAB, *X. albilineans* GyrA and GyrB. R and S, relaxed and supercoiled DNA, respectively. (C) Sensitivity of the hybrid EcA · XaB and *E. coli* DNA gyrase to ciprofloxacin and albicidin. R and S, relaxed and supercoiled DNA, respectively. EcA · XaB, *E. coli* GyrA and *X. albilineans* GyrB; EcAB, *E. coli* GyrA and GyrB.

The crystal structure of the C-terminal domain of GyrA from *E. coli* revealed a β-pinwheel fold with six propeller blades. The electropositive band around the perimeter of the pinwheel structure, at the outer edges of blades 4, 5, 6, and 1, is believed to be important in bending DNA into the required loop for negative supercoiling (13, 34). Therefore, it is interesting to see from alignment of the *X. albilineans* and *E. coli* domains that the two electronegative insertions in *X. albilineans* GyrA lie within loops that connect the fourth blade to the third and fifth blades, where they may modify this charged band. R691 is also interesting in this context because in *E. coli* GyrA, the corresponding residue D692, along with other conserved residues in the third propeller blade, has been shown to be important for DNA binding, supercoiling, and relaxation activities (20). These unusual electrochemical features of the *X. albilineans* GyrA do not cause distributive activity, as XaA · EcB is processive. However, they may be associated

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with the inability to relax supercoiled DNA, as discussed below.

Q83 is interesting because in *E. coli*, GyrA mutations S83L, S83W, and S83A confer 5- to 10-fold-increased quinolone resistance (11, 28) and S83W increases albicidin resistance by 4 to 6-fold (17). The *X. albilineans* GyrA has two other residues (T455 and Y664) that are uniquely variant from all other sequenced GyrA enzymes, but residues at these positions are not highly conserved and have not been previously implicated in gyrase function or antibiotic resistance.

The *X. albilineans* GyrB sequence includes the 12 residues important in ATP binding and hydrolysis, as revealed by the crystal structure of *E. coli* GyrB (41), and with two exceptions, it matches the *E. coli* sequence at residues implicated in antibiotic sensitivity. In *E. coli* GyrB, K447E confers quinolone resistance at the expense of an 80% reduction in supercoiling specific activity (18). The corresponding residue in *X. albilineans* GyrB is R463 (Fig. 2). In *E. coli* GyrB, R136C, R136H, R136L, R136A, and R136S confer resistance to coumarins (12, 14). The corresponding residue in *X. albilineans* GyrB is Q151. Although *X. albilineans* is susceptible to coumarins in vivo, the purified DNA gyrase from *X. albilineans* shows eightfold-higher resistance than the *E. coli* enzyme to novobiocin (Table 2). The six-amino-acid insertion in the N-terminal region (ATPase domain) of the *X. albilineans* GyrB (Fig. 2) may also be relevant in this context. A similar insertion exists in the same region in 10 sequenced GyrBs from *Chlamydia* spp., but the effect on coumarin sensitivity has not been reported.

Taken together, these considerations pointed toward an interaction between multiple sequence changes, most likely including GyrB Q151, GyrB R451, GyrA Q83, and the unusual insertion in the C-terminal region of the A subunit, resulting in a DNA gyrase conformation in *X. albilineans* that is less susceptible than its *E. coli* homologue to diverse antibiotics. The ability to reconstitute functional DNA gyrase in vitro by mixing the purified subunits provided an opportunity to resolve some of these contributions.

***The X. albilineans* GyrA subunit confers albicidin resistance.** The GyrA subunit from *X. albilineans* was compatible with GyrB from *E. coli.* The hybrid enzyme (XaA · EcB) equaled the *X. albilineans* holoenzyme (XaAB) in resistance to albicidin and ciprofloxacin, in contrast to the sensitivity of the *E. coli* holoenzyme (EcAB) (Table 2). Therefore, differences in the A subunit alone can account for the observed levels of albicidin and ciprofloxacin resistance, implying that the A subunit is the albicidin target. The molecular target was not resolved to this level in previous studies using *E. coli* mutants, because albicidin-resistance mutations in *E. coli* map to the *tsx* locus and block antibiotic uptake (6, 17).

***The X. albilineans* GyrA subunit is associated with an inability to relax supercoiled DNA.** Although relaxation of supercoiled DNA is energetically favorable and although this reaction by DNA gyrase is ATP independent, it is less efficient than ATP-dependent supercoiling, as evidenced by the higher enzyme concentrations required for relaxation assays (29). The mechanisms for the operation of the three gates in DNA gyrase during "reverse"-strand passage for relaxation are less well understood than those involving ATP for "forward"-strand passage during supercoiling (42).

The unexpected absence of detectable relaxation by the *X. albilineans* gyrase or the XaA · EcB hybrid may indicate a "tighter" gate which torsional energy in the supercoiled DNA is insufficient to operate. The evidence indicates that the difference arises as a result of the presence of the *X. albilineans* A subunit, which imparts local superhelicity to the looped DNA, contributes to the DNA cleavage gate, and forms the exit gate (in the supercoiling context).

The reciprocal hybrid (EcA · XaB) was not functional for supercoiling, but it was capable of relaxation in both the presence and the absence of ATP. Therefore, the EcA · XaB hybrid associates well enough for strand cleavage and "reverse"-strand passage through the three functional gates but evidently not well enough for complete transduction of the ATP-mediated conformational changes needed for supercoiling. The relaxation activity of the EcA · XaB enzyme was as sensitive as that of the *E. coli* holoenzyme to the presence of ciprofloxacin, which is consistent with the identification of the A subunit as the major determinant of this sensitivity.

Implications for development of new antibiotics and disease-resistant sugarcane. Because DNA gyrase is essential to prokaryotes, it is an appealing target for antibiotic development. Quinolones are the only commercially available pharmaceuticals that target DNA gyrase. However, resistance to quinolones resulting from single-base mutations in DNA gyrase genes has been documented for several clinically important pathogens. We have previously shown that albicidin is a potent inhibitor of DNA gyrase, with the unusual property of rapid, ATP-dependent stabilization of the gyrase-DNA cleavage complex (17). Here we show that the *X. albilineans* gyrase has multiple unique residues that, in combination, confer an unusual set of topoisomerase activities along with high-level resistance to diverse antibiotics. A deeper understanding of the unusual molecular interactions between albicidin and DNA gyrase may assist the effort to develop antibacterial agents that are more potent and last longer.

Albicidins are pathogenesis factors in sugarcane leaf scald disease, and albicidin resistance may be engineered to confer disease resistance (2, 46). Antibiotic resistance mediated by mutation in DNA gyrase is typically recessive (21) and therefore is probably not useful in efforts to devise a strategy for engineering a polyploid plant such as sugarcane. However, as in the albicidin-producing organism, it may be useful in plants to combine several mechanisms, such as a DNA-mimicking protein (17) along with an enzyme for albicidin detoxification (46), to obtain better protection against the disease.

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