Identification of a topoisomerase IV in actinobacteria: purification and characterization of ParY^R and GyrB^R from the coumermycin A₁ producer Streptomyces rishiriensis DSM 40489

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The biosynthetic gene clusters of the gyrase inhibitors coumermycin A₁ and clorobiocin contain two different resistance genes (gyrB^R and parY^R). Both genes code for B subunits of type II topoisomerases. The authors have now overexpressed and purified the encoded proteins, as well as the corresponding A subunits GyrA and ParX. Expression was carried out in Streptomyces lividans in the form of hexahistidine fusion proteins, allowing purification by nickel affinity chromatography. The complex of GyrA and GyrB^R was found to catalyse ATP-dependent supercoiling of DNA, i.e. to function as a gyrase, whereas the complex of ParX and ParY^R catalysed ATP-dependent decatenation and relaxation, i.e. the functions of topoisomerase IV (topo IV).

This is believed to represent the first topo IV identified in the class of actinobacteria, and the first demonstration of the formation of a topo IV as a resistance mechanism of an antibiotic producer.

INTRODUCTION

Bacterial DNA gyrase and topoisomerase IV (topo IV) are type II topoisomerases, involved in controlling the topological state of bacterial DNA by ATP-dependent reaction mechanisms (Maxwell & Lawson, 2003; Ullsperger & Cozzarelli, 1996). DNA gyrase, which is present in all known bacteria, has the unique ability to introduce negative supercoils into DNA, energetically driven by ATP hydrolysis. It is composed of the subunits GyrA and GyrB, which form a (GyrA)₂(GyrB)₂ heterotetramer. Topo IV is composed of the subunits ParC and ParE, which form a similar tetrameric structure (ParC)₂(ParE)₂. The best-examined function of topo IV is the decatenation of daughter chromosomes following DNA replication; in addition, topo IV relaxes superhelical DNA. The subunits GyrA and ParC contain the catalytic centre for DNA cleavage and rejoining, while the GyrB and ParE subunits contain the catalytic centre for ATP hydrolysis.

Most bacteria possess both these type II topoisomerases. However, a different situation is encountered in members of the class of actinobacteria: the genome sequences of coryneform bacteria (Corynebacterium glutamicum; Corynebacterium efficiens) and of mycobacteria (Mycobacterium tuberculosis; Mycobacterium leprae) contain only genes for the DNA gyrase subunits A (gyrA) and B (gyrB), whereas the complex of ParX and ParY catalyses ATP-dependent decatenation and relaxation, i.e. the function of topoisomerase IV (topo IV).

Recently, we have identified type II topoisomerase genes as resistance genes within the biosynthetic gene clusters of novobiocin, clorobiocin and coumermycin A₁ (Schmutz et al., 2003). These aminocoumarin antibiotics are potent inhibitors of the subunit B of gyrase (Maxwell, 1997), and are produced by different Streptomyces strains. These organisms protect their own gyrase from the inhibitory effect of the aminocoumarins by de novo synthesis of an aminocoumarin-resistant gyrase B subunit during antibiotic formation (Schmutz et al., 2003; Thiara & Cundliffe, 1988, 1989, 1993). The gene encoding this subunit (named gyrB^R) was found in all three gene clusters (Schmutz et al.,...
kanamycin (50 μg ml⁻¹) was used. Transformants of *S. lividans* TK24 with pUWL201 expression constructs (Schmutz et al., 2003) were selected with thiostrepton (50 μg ml⁻¹).

**PCR amplification and cloning of topoisomerase genes.** The primers used for PCR amplification of the topoisomerase genes are listed in Table 1. gyrB and parX were amplified from the cosmid 4-2H described previously (Schmutz et al., 2003). For amplification of the *S. coelicolor* genes gyrA and parX genomic DNA was used as template. Two different programmes for the amplification were used. For gyrB, parX and gyrA the conditions were 1 cycle at 95°C (5 min), followed by 30 cycles at 95°C (90 s), 60°C (90 s) and 72°C (5 min), and finally 1 cycle at 72°C (10 min). parX was amplified by denaturation at 96°C (5 min), followed by 30 cycles at 96°C (2 min) and 72°C (6 min), and finally 1 cycle at 72°C (10 min). Each 100 μl volume of reaction mixture contained about 100 ng template cosmid DNA or 2–5 μg genomic DNA, 20 pmol of each primer, 0–2 mM dNTPs (each, final concentration), Pfu DNA polymerase reaction buffer (2 mM MgSO₄, final concentration), 5% (v/v) DMSO, and 3 units of Pfu DNA polymerase (Promega). The PCR products were digested with the restriction enzymes given in Table 1 and ligated into pRSET B (Invitrogen) (resulting in pRSET B-gyrB, pRSET B-parX and pRSET B-gyrA, respectively) or pET-24a (+) (Novagen) (resulting in pET-24a-parX). To obtain plasmids replicating in *Streptomyces*, the pRSET B derivatives pRSET B-gyrB, pRSET B-parX and pRSET B-gyrA were digested with HindIII and fused with HindIII-digested pGM9. The construct pET-24a-parX was linearized with BglII and fused with BglII-digested pGM9.

**Complementation of gyrB and parE temperature-sensitive mutants of *E. coli*.** The *E. coli* strains N4177 (Menzel & Gellert, 1983) and W3110parE10 (Kato et al., 1990) contain either gyrB or parE with a mutation repressing the function of the resulting proteins at high temperature. Both strains were transformed either with pRSET B-gyrB or with pRSET B-parE. Transformed cells were grown on LB agar plates (Sambrook & Russell, 2001) containing carbenicillin (50 μg ml⁻¹) and IPTG (0.5 mM). As a positive control pAG111 containing gyrB from *E. coli* (Hallett et al., 1990) transformed into strain N4177 was used. Cells transformed with pAG111
were grown on LB agar plates supplemented with carbenicillin (50 µg ml⁻¹). One set of plates was incubated at 30 °C overnight, and the other was incubated at 42 °C overnight. Complementation was determined by the ability of the transformants to grow at 42 °C.

**Protein expression and purification.** *S. lividans* T7 strains harbouring the expression constructs were cultured in YEME medium (Kieser et al., 2000) supplemented with kanamycin (10 µg ml⁻¹) for 2 days at 28 °C in baffled flasks. Each flask containing 200 ml YEME medium, supplemented with kanamycin (10 µg ml⁻¹) and thiostrepton (25 µg ml⁻¹), was inoculated with 4 ml of the preculture. After 24 h cultivation at 28 °C (170 r.p.m. in baffled flasks with steel springs) cells were harvested by centrifugation (5000 g, 4 °C, 10 min) and frozen overnight at −70 °C. For lysis the cells were thawed and resuspended in 1 ml lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 8 mg lysozyme ml⁻¹) per g (wet wt) and incubated on ice for 30 min. Cells were broken by sonication (Branson Sonifier 250) for 10 min in 2 min intervals. The lysate was cleared by centrifugation (17 000 g, 4 °C, 30 min). The His-tagged proteins were purified by nickel affinity chromatography by using Ni-nitrilotriacetic acid resin (Qiagen). Some changes to the manufacturer’s standard protocol were necessary in the washing procedure to obtain nearly homogeneous protein fractions. The imidazole concentration in the washing buffer was increased to 30–40 mM, and in the case of GyrB®, urea (2 M) was added to remove traces of GyrA subunit. After elution of the recombinant proteins with 250 mM imidazole, the buffer was exchanged by gel filtration on Sephadex G-25 NAP-10 columns (Amersham Biosciences). The storage buffer contained 50 mM potassium phosphate, pH 7.5, 1 mM DTT, 0–2 mM EDTA and 50 % (v/v) glycerol. Refolding of denatured GyrB® protein was achieved by incubation overnight in storage buffer at 4 °C before storage at −20 °C.

The collected fractions were analysed by SDS-PAGE according to the method of Laemmli (1970) in 10 % gels.

**DNA gyrase and top IV activity assays.** DNA gyrase and top IV holoenzymes were reconstituted by mixing approximately equimolar amounts of recombinant GyrA and GyrB® and ParX and ParY® subunits in the reaction mixture, respectively. Enzyme activity was detected by incubation for 1 h at 30 °C in a total reaction volume of 20 µl containing 25 mM HEPES/KOH, pH 8.0, 10 mM magnesium acetate, 56 mM KCl, 2 mM DTT, 10 mM spermidine, 2 mM ATP and 50 µg BSA ml⁻¹. As substrates, 100 ng relaxed pBR322, 400 ng kinetoplast DNA (kDNA) (Topogen) and 100 ng supercoiled pBR322 were used for detection of supercoiling, decatenation and relaxation activity, respectively. The reaction was stopped by adding 4 µl loading buffer containing 200 mM EDTA, 30 % glycerol and 0–25 % bromphenol blue. Assays were analysed by electrophoresis on 0–8 % agarose gels in Tris/acetate/EDTA (TAE buffer) and staining with ethidium bromide. Electrophoresis with chloroquine was carried out on a 1–6 % agarose gel with 8–1 µg chloroquine phosphate ml⁻¹ (corresponding to 5 µg ml⁻¹ chloroquine base) in TAE buffer. Electrophoresis was performed in TAE buffer containing the same chloroquine concentration and run overnight. The gel was then washed three times for 30 min in 10 mM MgSO₄ in TAE buffer, and in distilled water before staining with ethidium bromide.

For testing the aminocoumarin resistance of supercoiling activity, 10–300 µg ml⁻¹ novobiocin (dissolved in buffer) was added to the incubation mixture. For testing the aminocoumarin resistance of decatenation activity, 2·5–100 µg ml⁻¹ novobiocin (dissolved in buffer) was added. IC₅₀ was defined as the novobiocin concentration causing 50 % inhibition of the supercoiling or of the decatenation reaction.

Supercoiled plasmid DNA of pBR322 was obtained by isolation from *E. coli* XL1 Blue MRF’ with ion-exchange columns (Nucleobond AX kits, Macherey-Nagel). Relaxation of supercoiled pBR322 was carried out with topoisomerase I (Amersham Biosciences) according to the manufacturer’s instructions.

**RESULTS**

**Complementation experiments with temperature-sensitive gyrB and parE mutants of *E. coli***

In *E. coli*, temperature-sensitive mutations affecting either gyrB or parE have been isolated (Kato et al., 1990; Menzel & Gellert, 1983). These mutants grow normally at 30 °C. If the temperature is raised to 42 °C, the function of the respective enzyme is repressed and the mutant stops growing, since both gyrase and top IV are essential for growth. Complementation of such mutants can be achieved with genes encoding a gyrase B subunit or a top IV ParE subunit, respectively, restoring growth at elevated temperature (Kato et al., 1992).

In order to test the function of gyrB® and parY® from the coumermycin cluster, both genes were cloned into the pRSET B vector. Each was transformed into the temperature-sensitive gyrB or parE mutants of *E. coli*. However, neither of the genes allowed growth at raised temperature. In contrast, transformation with a vector containing the respective

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<th>Primer</th>
<th>Nucleotide sequence (5’–3’)</th>
<th>Enzyme</th>
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<tr>
<td>gyrBC-1</td>
<td>GCCCCGAAAGAGCTCGAGTCGACTAC</td>
<td>Xho</td>
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<td>CGGTTCTCAGCTTACATGTCGAGGA</td>
<td>HindIII</td>
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<td>ACTCTCTAAGCTTCTAGCTGGCCCGA</td>
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topoisomerase gene from *E. coli* easily restored growth of the *E. coli* temperature-sensitive mutants at high temperature. This indicated that the GyrB<sup>R</sup> and ParY<sup>R</sup> proteins from *Streptomyces* did not form functional type II topoisomerasers with GyrA or ParC from *E. coli*, and therefore no conclusion could be drawn on the functions of GyrB<sup>R</sup> and ParY<sup>R</sup>.

**Expression and purification of GyrB<sup>R</sup>, ParY<sup>R</sup>, GyrA and ParX**

Attempts to purify GyrB<sup>R</sup> and ParY<sup>R</sup> as hexahistidine fusion proteins after expression in *E. coli* using the pRSET B vector were unsuccessful, since the proteins were contained in insoluble form in inclusion bodies. Therefore, *Streptomyces* strains were chosen for further expression experiments. GyrB<sup>R</sup> and ParY<sup>R</sup> were first expressed in *S. lividans* TK24, using the *Streptomyces* expression vector pUWL201. Affinity chromatography on novobiocin-Sepharose (Staudenbauer & Orr, 1981; Thiara & Cundliffe, 1988) allowed the purification of the genuine aminocoumarin-sensitive GyrB protein from *S. lividans*, which could be reconstituted with GyrA from the same organism to yield active gyrase. However, the aminocoumarin-resistant GyrB<sup>R</sup> and ParY<sup>R</sup> proteins did not bind to the affinity column and apparently eluted with the bulk of the proteins. Therefore, a different purification strategy had to be devised.

The pRSET B vectors constructed for expression in *E. coli* (see above) encoded hexahistidine fusion proteins of GyrB<sup>R</sup> and ParY<sup>R</sup>, suitable for purification by nickel affinity chromatography. In order to allow replication of these vectors and expression of GyrB<sup>R</sup> and ParY<sup>R</sup> in *S. lividans*, they were fused with the vector pGM9, which contains an origin of replication for *Streptomyces* (Muth et al., 1989).

The resulting vectors were introduced into *S. lividans* T7, which contains a thioestrepton-inducible gene for T7 RNA polymerase (Heinzelmann et al., 2001). After induction, crude protein extracts were prepared and subjected to nickel affinity chromatography. This resulted in nearly homogeneous ParY<sup>R</sup> protein, detected in the SDS gel at the expected molecular mass of 81 kDa (Fig. 2, lane 3). The GyrB<sup>R</sup> protein, however, appeared to form a very stable complex with GyrA of *S. lividans* T7 and therefore coeluted with GyrA (data not shown). Complete removal of GyrA was finally achieved by elution of the nickel affinity column with 2 M urea. GyrB<sup>R</sup> remained on the column and could subsequently be eluted with 250 mM imidazole, although in denatured form. Removal of imidazole and storage overnight at 4 °C resulted in the renaturation of GyrB<sup>R</sup>. The protein still contained two minor impurity bands (Fig. 2, lane 1), which however did not interfere with the assay.

The additional required subunits GyrA and ParX were cloned from the completely sequenced organism *S. coelicolor*, expressed in *S. lividans* T7 and purified by essentially the same procedure (see Methods), resulting in nearly homogeneous proteins (Fig. 2, lanes 2 and 4).

Investigation of supercoiling, deconcatenation and relaxation activities

Gyrase introduces negative supercoils into DNA, energetically driven by hydrolysis of ATP. Topo IV catalyses the catenation/deconcatenation of DNA and relaxes supercoiled DNA, with both reactions being dependent on the presence of ATP (Maxwell & Lawson, 2003).

Using relaxed and supercoiled pBR322 as well as catenated kinetoplast DNA, we investigated the supercoiling, relaxation and deconcatenation activity of the purified topoisomerase subunits in different combinations. As shown in Fig. 3(a) (lane 2), the complex of GyrA and GyrB<sup>R</sup> efficiently catalysed the supercoiling reaction. However, no ATP-dependent relaxation or deconcatenation was catalysed by this protein (lanes 2 in Fig. 3b, c), consistent with the expectation that the (GyrA)<sub>2</sub>(GyrB<sup>R</sup>)<sub>2</sub> complex functions as a gyrase.

On the other hand, the complex of ParX and ParY<sup>R</sup> showed clear relaxation and deconcatenation activity (lanes 3 in Fig. 3b, c), proving that these proteins function as subunits of topo IV. The subunits of gyrase and topo IV could not be interchanged: combinations of ParX with GyrB<sup>R</sup>, or of GyrA with ParY<sup>R</sup>, were not enzymically active in any of the three test systems (Fig. 3, lanes 4 and 5). All three observed reactions were dependent on the presence of ATP (Fig. 3, lanes 6).

From the experiment shown in Fig. 3(a) (lane 3), it appeared that the complex of ParX and ParY<sup>R</sup> showed a
low supercoiling activity. However, when the experiment was repeated and gel electrophoresis was performed in the presence of 5 µg chloroquine ml⁻¹, it was obvious that the complex of ParX and ParY↑ had no supercoiling activity (Fig. 4).

**Aminocoumarin resistance of GyrBR and ParY↑**

In our previous study (Schmutz et al., 2003), we had shown in vivo that gyrB↑ and parY↑ confer resistance to novobiocin and coumermycin A₁, i.e. that they are likely to encode aminocoumarin-resistant topoisomerase subunits. This was now investigated in vitro. The gyrase of *S. lividans* is highly sensitive to novobiocin, with an IC₅₀ value of approximately 0·5 µg ml⁻¹ (Thiara & Cundliffe, 1988), very similar to the IC₅₀ of *E. coli* gyrase, which was determined as approximately 0·6 µg ml⁻¹ (Peng & Marians, 1993). In contrast, we observed an IC₅₀ of 50 µg ml⁻¹ for the complex of GyrA and GyrBR. Likewise, bacterial topo IV is

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**Fig. 3.** DNA gyrase and topo IV activity assays: (a) supercoiling assay; (b) relaxation assay; (c) decatenation assay. Assay conditions were as described in Methods. In all assays equal amounts of the purified proteins were used. Assays were analysed by loading on a 0·8 % agarose gel and electrophoresis in TAE buffer. Gels were stained with ethidium bromide.

**Fig. 4.** Supercoiling and relaxation assay with DNA gyrase and topo IV. Each assay was divided and equal amounts were loaded on a 1 % agarose gel in TAE buffer (a) and a 1·6 % agarose gel with 5 µg chloroquine ml⁻¹ in TAE buffer (b). Both gels were stained with ethidium bromide.
sensitive to aminocoumarins (Hardy & Cozzarelli, 2003; Peng & Marians, 1993), with an IC$_{50}$ of 1.5 µg ml$^{-1}$ reported for the (ParC)$_2$(ParE)$_2$ complex of E. coli (Peng & Marians, 1993). In contrast, the (ParX)$_2$(ParYR)$_2$ complex investigated in this study showed an IC$_{50}$ of 20 µg ml$^{-1}$ in the decatenation assay.

**DISCUSSION**

In this study, we present for the first time evidence for the existence of a topo IV in actinobacteria. Mycobacteria and corynebacteria possess only a single type II topoisomerase, which apparently has both supercoiling and decatenating activity (Manjunatha et al., 2002). We have shown that streptomycetes (which also belong to the actinobacteria) contain both a gyrase, which consists of GyrA and GyrB subunits and catalyses the supercoiling of DNA, and a topo IV, consisting of the ParX and ParY subunits and catalysing the ATP-dependent decatenation and relaxation of DNA.

The topo IV which was investigated in the present study in vitro and in our previous study in vivo (Schmutz et al., 2003) contained the ParX subunit of S. coelicolor and the ParYR subunit from the coumermycin biosynthetic gene cluster of S. rishiriensis. This demonstrates the compatibility of the topo IV subunits from different Streptomyces strains. In the same way, the compatibility of the gyrase subunits A and B from different strains was shown. In contrast, the failure to complement E. coli gyrB and parE mutants with gyrB$^R$ and parE$^R$ from Streptomyces indicated that the Streptomyces proteins may not form functional topoisomerases with their counterparts from E. coli, as observed previously (Simon et al., 1995). However, our results do not rule out the possibility of a partial complementation, which may be observable under less stringent conditions.

Genome sequencing of S. coelicolor and Bifidobacterium longum has demonstrated in each organism the presence of a gyrA and a gyrB gene as well as of a parX and a parY gene (Figs 1 and 5). For both organisms, the genes corresponding to parX and parY are at present annotated as ‘putative DNA gyrase subunits’ in the database. Our study has now shown that these genes encode a topo IV rather than a gyrase.

In the genomes of most Gram-positive bacteria, parE and parC are immediately adjacent genes, probably transcribed as a single operon, as observed for gyrB and gyrA (Fig. 5). In contrast, in S. coelicolor and in B. longum, parX and parY have opposite orientations and are separated by a stretch of 13 and 8 kb, respectively. Therefore parX and parY are regulated by different promoters. A similar situation is encountered in Streptomyces avermitilis, which has recently been sequenced (Omura et al., 2001).

gyrB$^R$ and parY$^R$ from the coumermycin biosynthetic gene cluster in S. rishiriensis protect this organism from the toxic effect of its own antibiotic. A gyrB$^R$ gene is also found in the biosynthetic gene cluster of novobiocin (Steffensky et al., 2000), and the function of this gene and the regulation of its expression has been investigated by Thiara & Cundliffe (1988, 1989, 1993). The fact that the coumermycin cluster contains not only a gyrB$^R$ gene but also a parY$^R$ gene (which is absent in the novobiocin cluster) is consistent with the very high affinity of coumermycin A$_1$ both for gyrase and for topo IV (Peng & Marians, 1993),

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**Fig. 5.** Genomic organization of gyrase and topo IV genes in Gram-positive bacteria.
creating the need to efficiently protect both enzymes in the coumermycin producer. This finding gives additional support to the hypothesis that topo IV is a biological target of coumermycin A1, as well as of clorobiocin, since the biosynthetic gene cluster of the latter antibiotic contains both gyrB and parY resistance genes (Schmutz et al., 2003).

Present evidence suggests that for gyrase inhibitors of the fluoroquinolone type, gyrase is the primary target in Gram-negative organisms, but topo IV is the primary target in Gram-positive organisms (Blanche et al., 1996). In contrast, for gyrase inhibitors of the aminocoumarin type, gyrase appears to be the primary target both in Gram-negative and in Gram-positive organisms, with topo IV representing a secondary target (Blanche et al., 1996; Hardy & Cozzarelli, 2003). The high affinity of coumermycin and clorobiocin to both their primary and secondary targets may explain the need of the antibiotic producers to create a resistance mechanism protecting both targets, which is realized by the expression of the Gyrb and ParY proteins.

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