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Resistance Genes of Aminocoumarin Producers: Two Type II Topoisomerase Genes Confer Resistance against Coumermycin A_1 and Clorobiocin

Elisabeth Schmutz, Agnes Mühlenweg, Shu-Ming Li, and Lutz Heide*

Pharmazeutische Biologie, Eberhard-Karls-Universität Tübingen, D-72076 Tübingen, Germany

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The aminocoumarin resistance genes of the biosynthetic gene clusters of novobiocin, coumermycin A_1, and clorobiocin were investigated. All three clusters contained a *gyrB*^{R} resistance gene, coding for a gyrase B subunit. Unexpectedly, the clorobiocin and the coumermycin A_1 clusters were found to contain an additional, similar gene, named *parY*^{R}. Its predicted gene product showed sequence similarity with the B subunit of type II topoisomerases. Expression of *gyrB*^{R} and likewise of *parY*^{R} in *Streptomyces lividans* TK24 resulted in resistance against novobiocin and coumermycin A_1, suggesting that both gene products are able to function as aminocoumarin-resistant B subunits of gyrase. Southern hybridization experiments showed that the genome of all three antibiotic producers and of *Streptomyces coelicolor* contained two additional genes which hybridized with either *gyrB*^{R} or *parY*^{R} and which may code for aminocoumarin-sensitive GyrB and ParY proteins. Two putative transporter genes, *novA* and *couR5*, were found in the novobiocin and the coumermycin A_1 cluster, respectively. Expression of these genes in *S. lividans* TK24 resulted in moderate levels of resistance against novobiocin and coumermycin A_1, suggesting that these genes may be involved in antibiotic transport.

The aminocoumarin antibiotics novobiocin, clorobiocin, and coumermycin A_1 (Fig. 1A) are known as potent inhibitors of gyrase (18). Their equilibrium dissociation constants are in the range of 10 nM (10), i.e., their affinity for gyrase is considerably higher than that of modern fluoroquinolones. Novobiocin is licensed as an antibiotic for clinical use (Albamycin; Pharmacia-Upjohn) and is used for the treatment of infections with multiresistant gram-positive bacteria, e.g., *Staphylococcus aureus*.

Novobiocin is produced by *Streptomyces spheroides* (synonym *S. caeruleus* [15]) NCIMB 11891, clorobiocin is produced by *S. roseochromogenes* var. *oscitans* DS12.976, and coumermycin A_1 is produced by *S. rishiriensis* DSM 40489 (2). Obviously, these organisms must protect their gyrases from the inhibitory effect of aminocoumarin during antibiotic formation. Thiara and Cundliffe (29–31) reported that the principal resistance mechanism of the novobiocin producer *S. sphaeroides* is the de novo synthesis of a coumarin-resistant gyrase B subunit, which replaces the sensitive GyrB subunit in the active $(GyrA)_2(GyrB)_2$ heterotetramer. Thus, this novobiocin producer contains two *gyrB* genes, a constitutively expressed *gyrB*^{S}, encoding the coumarin-sensitive protein, and the *gyrB*^{R} gene, encoding the resistant protein and expressed in the presence of novobiocin. The promoter of *gyrB*^{R} appears to be regulated by changes in the superhelical density of DNA (30).

Mitchell et al. (20) supplied evidence that additional genes may contribute to novobiocin resistance. They used the novobiocin producer *S. niveus*, which has recently been identified as a subjective synonym for *S. spheroides* (15).

We cloned and sequenced the novobiocin biosynthetic gene cluster (27), depicted in Fig. 1B. On its right border, the cluster contains the *gyrB*^{R} resistance gene. Near the left border, the gene *novA*, encoding an ABC transporter, was identified. Méndez and Salas (19) suggested that *novA* is involved in transport of and possibly resistance against novobiocin. They classified the encoded protein as a type III ABC transporter, i.e., the ATP binding domain and the membrane domain are fused together on the same protein chain.

Recently our group has also cloned and sequenced the core regions of the biosynthetic gene clusters of coumermycin A_1 (35) and of clorobiocin (23) (Fig. 1B). The present study was undertaken in order to identify and compare putative aminocoumarin resistance genes in the three aminocoumarin clusters.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Strains and plasmids used in this study are listed in Table 1. *S. lividans* TK24 was cultured at 28°C and 170 rpm for 2 to 4 days in baffled shake flasks in HA medium containing 1.0% malt extract, 0.4% yeast extract, 0.4% glucose, and 1.0 mM $CaCl_2$ (pH 7.3). For preparing protoplasts of *S. lividans* TK24, CRM medium containing 10.3% sucrose, 2.0% tryptic soy broth, 1.0% $MgCl_2$, 1.0% yeast extract, and 0.75% glycine (pH 7.0) was used. *Streptomyces* protoplasts were prepared and transformed as described before (14). Regeneration of protoplasts was carried out on R2YE medium (14). For selection of thiostrepton-resistant strains of *S. lividans* TK24, HA agar plates containing 50 μg of thiostrepton per ml were used.

DNA isolation and manipulation. Standard methods were used for DNA isolation and manipulation in *Escherichia coli* XL1 Blue MRF′ (25). DNA fragments were isolated from agarose gels with a QIAquick gel extraction kit according to the instructions of the manufacturer.

Construction of plasmids for heterologous gene expression in *S. lividans* TK24. From the novobiocin biosynthetic gene cluster, *gyrB*^{R} and *novA* were cloned for heterologous expression in *S. lividans* TK24. A 2.3-kb *Apa*I-*Pst*I fragment from plasmid p10-9CE2 containing *gyrB*^{R} was isolated and ligated into the same sites of Litmus 38. The resulting construct was digested with *Apa*I and *Eco*RI, and the 2.3-kb fragment was introduced into pGEM-11Zf(+). The insert of the plasmid obtained was excised with *Hin*dIII and *Eco*RI and cloned into the corresponding sites of pUWL201 to give the expression plasmid pGES1$^+$.

* Corresponding author. Mailing address: Eberhard-Karls-Universität Tübingen, Pharmazeutische Biologie, Auf der Morgenstelle 8, D-72076 Tübingen, Germany. Phone: 49 7071 2978789. Fax: 49 7071 295250. E-mail: heide@uni-tuebingen.de.

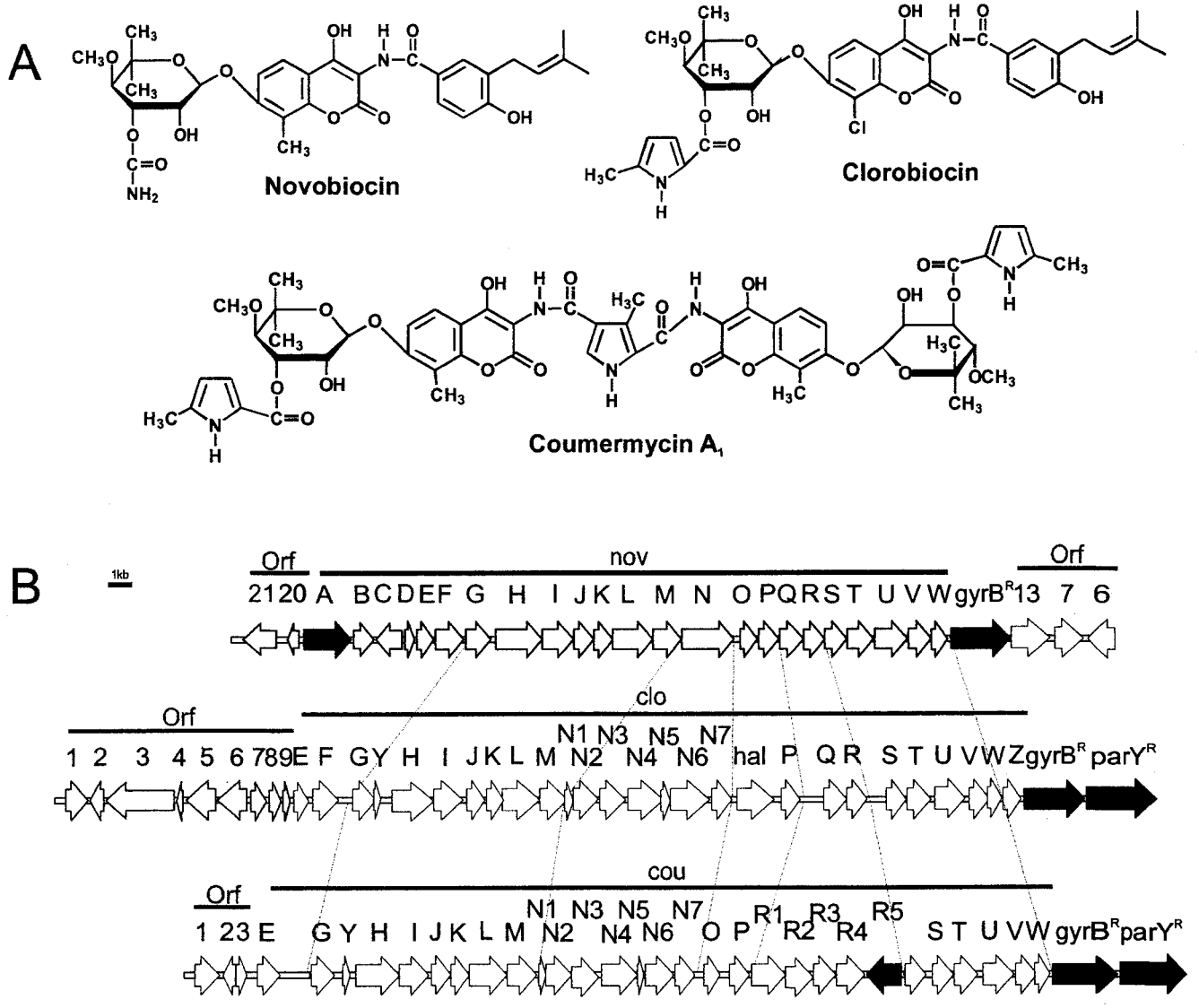

FIG. 1. (A) Structures of the aminocoumarin antibiotics. (B) Gene clusters of novobiocin (top), clorobiocin (middle), and coumermycin A_1 (bottom). Genes involved in aminocoumarin resistance are shown by solid arrows.

novA was isolated from pMS63 by digestion with *Sna*BI and *Pst*I (2.05 kb) and cloned into the *Eco*RV and *Pst*I sites of pBluescript SK(−). After digestion with *Hin*dIII and *Pst*I, *nov*A was ligated into the corresponding sites of pUWL201. The resulting expression plasmid was named pTES3.

From the coumermycin A_1 biosynthetic gene cluster, the three genes $gyrB^R$, $parY^R$, and *couR5* were cloned for expression experiments. The 2.2-kb *Pvu*II-*Mlu*I fragment of the cosmid 4-2H containing $gyrB^R$ was ligated into the *Eco*RV and *Bss*HII sites of Litmus 28. The insert was excised with *Hin*dIII and *Spe*I and cloned into pUWL201 to give the expression plasmid pGES2. Cosmid 4-2H was also digested with *Bam*HI and *Xho*I, and a 2 kb-fragment containing the C terminus of $parY^R$ was cloned into the same sites of pGEM-11Zf(+) to give pGES31. A fragment encoding the N-terminal region of $ParY^R$ was amplified by PCR with cosmid 4-2H as template. The synthetic oligonucleotides used for the amplification were primer gyrBX-1 (GCCCCTCTAGACGCGTGCGTGACCC AAAG) and primer gyrBX-2 (GATGACCTCGATGTGGTCGCAGGCAC); an *Xba*I site (underlined) was introduced into primer gyrBX-1. The PCR fragment was cloned into the *Xba*I and *Bam*HI sites of pGES31. The plasmid containing the complete $parY^R$ gene was digested with *Hin*dIII and *Eco*RI, and the resulting fragment was ligated into the corresponding sites of pUWL201, forming the expression plasmid pGES3.

To generate a construct for coexpression of $gyrB^R$ and $parX^R$, pGEM-11Zf(+) was digested with *Nsi*I and treated with Klenow fragment, creating a blunt end. The vector was then digested with *Not*I and ligated with a 3.44-kb *Pvu*II-*Not*I fragment isolated from cosmid 4-2H to give pGES41. Subsequently, a 0.87-kb *Not*I-*Xho*I fragment was also isolated from cosmid 4-2H and ligated into the corresponding sites of pGES41. The insert containing $gyrB^R$ and $parY^R$ was excised with *Hin*dIII and *Eco*RI and ligated into pUWL201 generating the expression construct pGES4.

couR5 was cloned into pGEM-3Zf(+) as a 1.56-kb *Sma*I-*Acc*I fragment from pZW10. Then the insert was excised with *Hin*dIII and *Pst*I and cloned into the same sites of pUWL201 to give the expression plasmid pTES4.

The constructs were introduced into *S. lividans* TK24, a *Streptomyces* strain which is very closely related to *S. coelicolor* and which is commonly used for expression experiments by protoplast transformation. Transformants were selected by thiostrepton resistance, and the presence of the intact expression construct was confirmed by plasmid isolation and restriction analysis (data not shown).

Novobiocin and coumermycin A_1 susceptibility testing. About 10^6 spores of *S. lividans* TK24 containing the expression plasmids $pGES1^+$, pGES2, pGES3, pGES4, pTES3, or pTES4 were plated on minimal medium (14) containing 20 μg of thiostrepton per ml and different concentrations of novobiocin or coumermycin A_1, respectively. Growth was determined after 6 days of incubation at 30°C.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
Streptomyces lividans TK24	Streptomycin resistant, no plasmids	14
Escherichia coli XL1 Blue MRF′	Tet^r, host strain for cloning experiments	Stratagene
Plamids and cosmids		
Litmus 28	Cloning vector, Amp^r	NE BioLabs
Litmus 38	Cloning vector, Amp^r	NE BioLabs
pBluescript SK(−)	Cloning vector, Amp^r	Stratagene
pGEM-3Zf(−)	Cloning vector, Amp^r	Promega
pGEM-11Zf(+)	Cloning vector, Amp^r	Promega
pUWL201	*E. coli-Streptomyces* shuttle vector, Amp^r Tsr^r *ermE*p* promoter	27
10-9C	Cosmid isolated from *S. spheroides* genomic DNA library	27
p10-9CE2	4.3-kb *Eco*RI fragment from 10-9C containing *gyrB^R* in pBluescript SK(−)	This work
4-2H	Cosmid isolated from *S. rishiriensis* genomic DNA library	35
pMS63	11.8-kb *Not*I-*Hin*dIII fragment from novobiocin biosynthetic gene cluster containing *novA* in pBluescript SK(−)	This work
pZW10	12.75-kb *Bgl*II fragment (bp 16013 to 28761 from AF 235050) from coumermycin A_1 biosynthetic gene cluster in pBluescript SK(−)	This work

Southern hybridization. A 0.9-kb *Bgl*II fragment (bp 1012 to 1923 of AF205853) containing *gyrB^R* and a 1.33-kb *Bgl*II-*Pvu*II fragment (bp 3271 to 4600 of AF205853) containing a part of the *parY^R* gene from *S. rishiriensis* were labeled with the Digoxigenin High Prime DNA labeling and detection starter kit II (Roche Applied Science, Mannheim, Germany) and used as probes for Southern blot analysis on Hybond-N nylon membranes (Amersham Biosciences, Freiburg, Germany). Genomic DNA from *Streptomyces spheroides* NCIMB 11891, *S. roseochromogenes* var. *oscitans* DS12.976, *S. rishiriensis* DSM 40489, and *S. coelicolor* A3(2) was isolated as described before (14).

Sequence analysis. Double-stranded sequencing was performed by the dideoxynucleotide chain termination method on a LI-COR automatic sequencer (MWG-Biotech AG, Ebersberg, Germany).

Database searches were performed in the GenBank database with the BLAST 2.0 program. DNASIS (version 2.1, 1995; Hitachi Software Engineering) was used for computer-aided sequence analyses.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper were deposited in the GenBank nucleotide sequence database under accession numbers AF205854, AF205853, and AY136281.

RESULTS

Identification and sequence analysis of type II topoisomerase genes. Our previous sequencing of the biosynthetic gene clusters of novobiocin, clorobiocin, and coumermycin A_1 (23, 27, 35) revealed the presence of a *gyrB^R* gene towards the right end of all three clusters (Fig. 1), each presumably coding for a coumarin-resistant GyrB subunit. We now extended the sequencing at the borders of the clusters (Fig. 1B). This revealed that both the coumermycin A_1 and the clorobiocin clusters contained a gene immediately downstream of *gyrB^R* which showed, on average, 44% identity and 57% similarity (amino acid level) to *gyrB^R* of the same organism, suggesting that it may encode a type II topoisomerase B subunit containing a well-conserved ATP binding site. We suggest the name *parY^R* for these two genes. No *parY^R* homologue was found in the novobiocin cluster.

The promoter regions of *gyrB^R* showed about 75% sequence identity (nucleotide level) between the three clusters, indicating that expression of *gyrB^R* in all these strains may be regulated in a similar way, as described by Thiara and Cundliffe (30). The intergenic region between *gyrB^R* and *parY^R* comprised 79 bp and 80 bp in *S. rishiriensis* and *S. roseochromogenes*, respectively. Both organisms contained a well-conserved ribosome binding site (AGGAG) 8 bp upstream of the *parY^R* start codon. No evidence was found for the presence of a transcription terminator or for sequence similarity to common bacterial promoters. This may indicate that *gyrB^R* and *parY^R* are transcribed as a single operon.

The sequence of *gyrB^R* of *S. spheroides* identified in our study was not identical to that reported previously by Thiara and Cundliffe (EMBL Z17304). However, the sequence published by these authors and their restriction map given for the DNA region surrounding the *gyrB^R* gene (31) were in excellent agreement with the *gyrB^R* region of *S. rishiriensis*. We reconfirmed the identity of the strains used in our laboratory by ordering new strain samples from the respective culture collections and by identification of the antibiotics produced by all strains by high-pressure liquid chromatography and by mass spectroscopy in comparison to authentic standards. This procedure confirmed the identity of all our strains. The most plausible explanation for these findings is that the sequence which Thiara and Cundliffe (31) examined was derived from *S. rishiriensis* rather than from *S. spheroides*.

In the three clusters, the *gyrB^R* genes were equal in size (coding for proteins of 677 amino acids) and showed a sequence identity of approximately 92% on the amino acid level. They showed, on average, 75% identity to the *gyrB* gene (SCO3874) of *S. coelicolor* A3(2), the genome of which has recently been sequenced, and 41% identity to the *gyrB* genes of other gram-positive bacteria, such as *Bacillus subtilis*, *Staphylococcus aureus*, and *Clostridium perfringens* (Fig. 2B).

The two *parY^R* genes coded for proteins of 702 amino acids with 91% sequence identity. A very similar gene exists in the genome of *S. coelicolor* (87% identity, amino acid level), SCO5822, which has been annotated as a putative DNA gyrase subunit B in the database and which has not yet been functionally identified. This gene of *S. coelicolor* will be referred to as *parY* hereafter.

Many bacteria contain two type II topoisomerases, i.e., a gyrase encoded by the genes *gyrA* and *gyrB* and a topoisomerase IV encoded by the genes *parC* and *parE* (34). It is not possible to distinguish gyrase and topoisomerase IV unambiguously by characteristic sequence motifs, although sequence comparison with functionally identified topoisomerases in re-

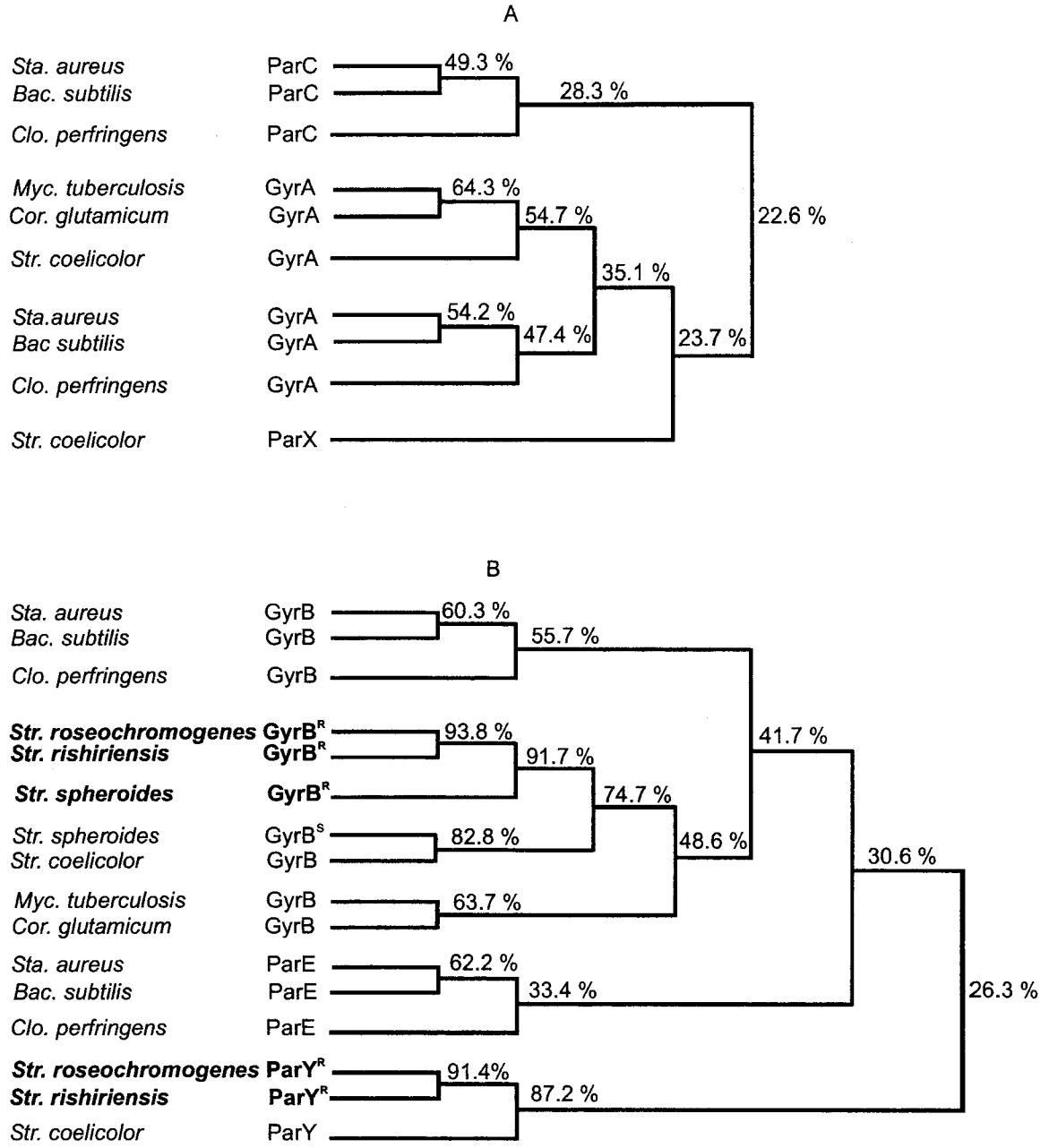

FIG. 2. Phylogenetic trees based on sequence similarities of the genes coding for the A subunits (A) and the B subunits (B) of type II topoisomerases in gram-positive bacteria. Resistance genes of the aminocoumarin antibiotic producers are shown in boldface. The phylogeny was constructed with DNASIS for Windows, version 2 (Hitachi, San Bruno), scoring with a gap penalty of 5.0, a K-tuple of 2.0, a fixed gap penalty of 10.0, and a floating gap penalty of 10.0. The number of top diagonals and window size were both set 5. Bacterial strains: Sta., *Staphylococcus*; Bac., *Bacillus*; Clo., *Clostridium*; Myc., *Mycobacterium*; Cor., *Corynebacterium*; Str., *Streptomyces*.

lated organisms usually allows classification into one of the two groups. This was, however, not possible for the *parY* genes; in a sequence comparison to the known *gyrB* and *parE* genes of gram-positive bacteria (Fig. 2B), the *parY* genes formed a group on their own, with only around 26% amino acid sequence identity to the products of the *gyrB* and *parE* genes.

Expression of *gyrB*^{R} and *parY*^{R} genes. In order to investigate the role of the *gyrB*^{R} and the *parY*^{R} genes in aminocoumarin resistance, we expressed *gyrB*^{R} and *parY*^{R} of *S. rishiriensis* in *Streptomyces lividans* TK24, with *gyrB*^{R} from *S. spheroides* as the comparison. All three genes were cloned separately into the *Streptomyces* expression vector pUWL201 (see Materials and Methods), which contains the constitutive *ermE*p* promoter for foreign gene expression and a thiostrepton resistance marker. Table 2 shows the growth inhibition caused by different antibiotic concentrations in the transformants. In a control strain transformed with the empty vector pUWL201, complete growth inhibition was achieved with 100 μg of novo-

biocin and 50 μg of coumermycin A_1 per ml. The higher antibacterial activity of coumermycin A_1 compared to novobiocin has been reported previously (12, 22).

The *gyrB*^{R} genes from the novobiocin producer *S. spheroides* and from the coumermycin A_1 producer *S. rishiriensis* clearly provided resistance, with complete inhibition achieved with 500 μg of coumermycin A_1 and >750 μg of novobiocin per ml. Both genes provided equally effective protection against novobiocin and against coumermycin A_1, proving that they encode general aminocoumarin resistance rather than specific protection from either of these two structurally different antibiotics.

Likewise, the newly discovered *parY*^{R} gene clearly provided resistance against both novobiocin and coumermycin A_1, although the resistance level was somewhat lower than that produced by the *gyrB*^{R} genes, with complete inhibition achieved with 300 μg of coumermycin A_1 and 500 μg of novobiocin per ml (Table 2).

In an additional experiment, we placed the entire sequence comprising both *gyrB*^{R} and *parY*^{R} from *S. rishiriensis* into the pUWL201 expression vector. Upon expression in *S. lividans* TK24, this construct led to the same level of resistance as expression of *gyrB*^{R} alone (Table 2). Since *gyrB*^{R} and *parY*^{R} are presumably transcribed as a single operon (see above), this result indicates that the effects of *gyrB*^{R} and *parY*^{R} expression are not additive or synergistic under the experimental conditions of our study.

A attempt to purify $GyrB^R$ and $ParY^R$ and to investigate their activity in vitro remained unsuccessful. Expression of fusion proteins of $GyrB^R$ and $ParY^R$ with N-terminal His_6 tags from the pRSET B vector system in *E. coli* yielded high expression, but only in the form of insoluble inclusion bodies. Varying the cultivation temperature between 37°C and 15°C, the inducer concentrations (50 μM to 500 μM), and the time of induction did not help to alleviate this problem.

Obviously, *gyrB*^{R} and *parY*^{R} had been actively expressed from the pUWL201 constructs in *S. lividans* TK24, as demonstrated by the resulting aminocoumarin resistance. We therefore tried to purify the $GyrB^R$ and $ParY^R$ proteins from these strains by affinity chromatography on novobiocin-Sepharose and elution with different concentrations of KCl and urea (26, 29). This resulted in successful purification of the native coumarin-sensitive gyrase B subunit of *S. lividans* TK24, which could be reconstituted with the GyrA subunit to an enzymatically active, supercoiling enzyme. However, we did not succeed in isolating the coumarin resistance enzymes. Apparently, these proteins did not bind to the novobiocin-Sepharose and eluted together with the bulk of the proteins. Attempts to determine gyrase activity in crude extracts without purification were unsuccessful.

Hybridization experiments with *gyrB*^{R} and *parY*^{R}. Thiara and Cundliffe (30) reported that the novobiocin producer *S. spheroides* is able to express a novobiocin-resistant $GyrB^R$ protein in addition to the novobiocin-sensitive $GyrB^S$ protein. It was likely that the same strategy was employed by the producers of clorobiocin and coumermycin A_1. The same scenario may be encountered for the *parY* genes, i.e., we speculate that the genomes of *S. roseochromogenes* and *S. rishiriensis* may contain an additional *parY*^{S} gene besides the *parY*^{R} genes located in the clorobiocin and coumermycin A_1 biosynthetic gene clusters.

TABLE 2. Growth of *S. lividans* TK24 harboring resistance genes from *S. spheroides* (Ss) and *S. rishiriensis* (Sr)

Gene(s)	Growth[a] with novobiocin at (μg/ml):						Novobiocin MIC (μg/ml)	Growth with coumermycin A_1 at (μg/ml):					Coumermycin A_1 MIC (μg/ml)
	0	50	100	250	500	600	750		0	50	100	250	300	500	
Control (pUWL201)	+	+	0	0	0	0	0	100	+	0	0	0	0	0	50
Ss *gyrB*^{R}	+	+	+	+	+	+	+	>750	+	+	+	+	+	0	500
Sr *gyrB*^{R}	+	+	+	+	+	+	+	>750	+	+	+	+	+	0	500
Sr *parY*^{R}	+	+	+	+	0	0	0	500	+	+	+	+	0	0	300
Sr *gyrB*^{R} + *parY*^{R}	+	+	+	+	+	+	+	>750	+	+	+	+	+	0	500
Ss *novA*	+	+	+	+	0	0	0	500	+	+	+	+	0	0	250
Sr *couR5*	+	+	+	0	0	0	0	250	+	+	0	0	0	0	250

[a] +++++, more than 3,000 colonies; ++++, 1,000 to 3,000 colonies; +++, 500 to 1,000 colonies; ++, 100 to 500 colonies; +, 10 to 100 colonies; 0, no growth observed. MIC, minimum concentration causing complete growth inhibition under the conditions of this experiment.

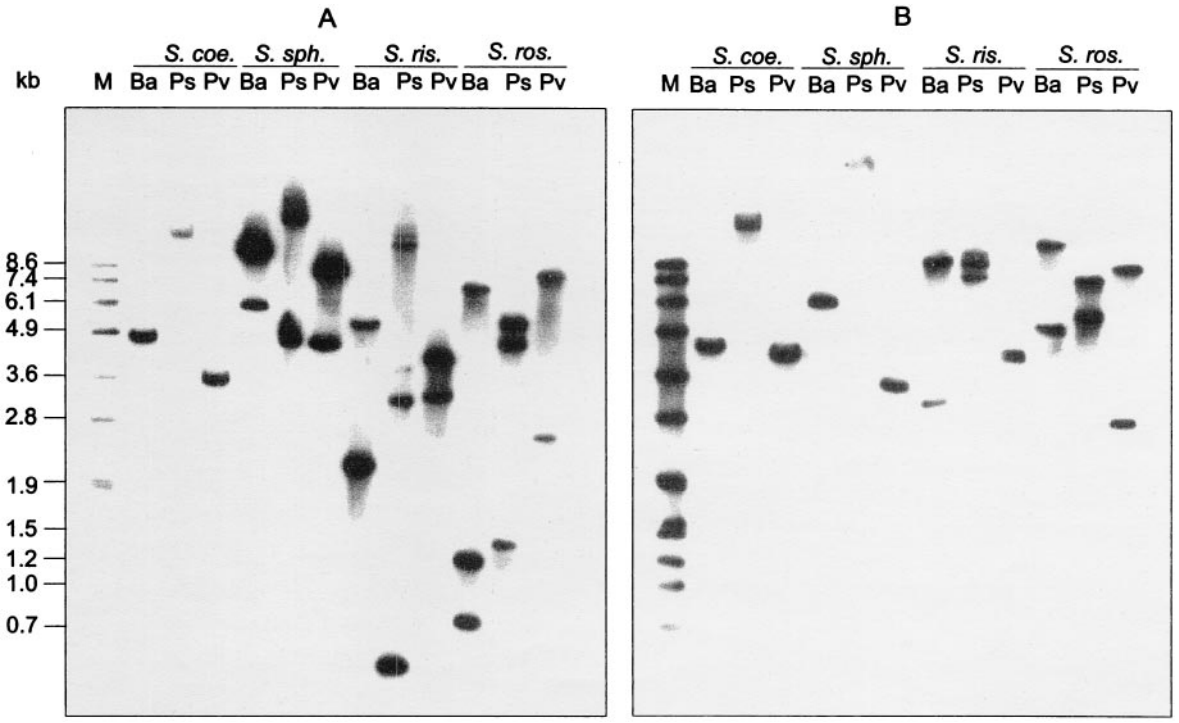

FIG. 3. Southern blotting of genomic DNA of *S. coelicolor* (*S. coe.*), *S. spheroides* (*S. sph.*), *S. rishiriensis* (*S. ris.*), and *S. roseochromogenes* (*S. ros.*). Genomic DNA was digested with *Bam*HI (Ba), *Pst*I (Ps), and *Pvu*II (Pv). $gyrB^R$ (A) and $parY^R$ (B) from *S. rishiriensis* were used as hybridization probes (see Materials and Methods). In blot B, *S. rishiriensis* DNA digested with *Pvu*II showed two bands at 4.1 and 4.3 kb, which are not clearly resolved in this figure.

In order to investigate the number of *gyrB* and *parY* genes in these organisms, we conducted Southern hybridization experiments with $gyrB^R$ and $parY^R$ from *S. rishiriensis* as probes. The high sequence similarity within the *gyrB* genes and within the *parY* genes, as well as the sequence differences between *gyrB* and *parY* (see Fig. 2B), suggested that selective hybridization for each group was possible. Therefore, genomic DNA from the producers of novobiocin, clorobiocin, and coumermycin A_1 as well as from the completely sequenced organism *S. coelicolor* A3(2) was digested with different enzymes and hybridized to the two probes consecutively. The resulting blots are shown in Fig. 3. As expected, the $gyrB^R$ probe of *S. rishiriensis* hybridized with the *gyrB* gene from *S. coelicolor*, resulting in a single band in each restriction digest. The observed bands coincided with the fragment size calculated from the genomic sequence (*Bam*HI, 4,496 bp; *Pst*I, 11,668 bp; *Pvu*II, 3,453 bp). In contrast, two bands hybridizing with $gyrB^R$ were detected in *S. spheroides*, similar to the genes $gyrB^R$ and $gyrB^S$ described by Thiara and Cundliffe (31). Likewise, two *gyrB* genes were detected in both *S. roseochromogenes* and *S. rishiriensis*. The $gyrB^R$ gene of *S. rishiriensis* contains a *Pst*I restriction site, and the $gyrB^R$ gene of *S. roseochromogenes* contains both a *Pst*I and a *Bam*HI site. This led to the appearance of three bands in the respective lanes (Fig. 3A).

With the $parY^R$ probe from *S. rishiriensis*, the *parY* gene of *S. coelicolor* was again detected, with the correct fragment sizes as calculated from the genomic sequence (*Bam*HI, 4,183 bp; *Pst*I, 13,021 bp; *Pvu*II, 3,965 bp). Also in *S. spheroides*, a single gene hybridizing to the $parY^R$ probe was detected. This suggests that the novobiocin producer, like *S. coelicolor*, also contains just one *parY* gene in its genome, since the novobiocin biosynthetic gene cluster does not contain a $parY^R$ resistance gene.

In both *S. roseochromogenes* and *S. rishiriensis*, two bands hybridizing with $parY^R$ were detected, suggesting that these organisms contain an additional *parY* gene besides the $parY^R$ genes identified in the biosynthetic gene clusters of clorobiocin and coumermycin A_1.

Expression of putative transporter genes *novA* and *couR5*. The gene *novA*, located near the left border of the novobiocin biosynthetic gene cluster, showed sequence similarity to type III ABC transporters (19), such as the ABC transporters found in the biosynthetic gene clusters of complestatin (4) and chloroeremomycin (33). The characteristic Walker motifs within the C-terminal part of the protein are especially well preserved. Méndez and Salas (19) suggested that the *novA* gene product may be involved in the transport of and/or resistance against novobiocin, but no experimental evidence has been provided so far. Our sequencing of the clorobiocin and the coumermycin A_1 clusters revealed no similar ABC transporter within or near these clusters (Fig. 1B). However, the coumermycin A_1 cluster contained the gene *couR5*, which showed sequence similarity to transmembrane efflux proteins presumably involved in antibiotic transport, e.g., to the putative actinorhodin transporter from *S. coelicolor* (8) and to the putative tetracenomycin resistance protein from *S. glaucescens* (11).

In order to investigate the possible role of *novA* and *couR5* in aminocoumarin resistance, we expressed both genes in *S. lividans* TK24 with the same expression vector, pUWL201, and

TABLE 3. Alignment of amino acids involved in aminocoumarin binding by *E. coli* gyrase, with corresponding positions of type II topoisomerase B subunits of different bacteria[a]

Species	Protein	Size (aa)	Position									
E. coli	GyrB	804	Val 43	Asn 46	Glu 50	Asp 73	Gly 77	Ile 94	Val 120	**Arg 136**	**Gly 164**	Thr 165
Novobiocin interaction			+	+	+	+	−	−	−	+	−	−
Clorobiocin interaction			+	+	+	+	+	−	−	+	−	+
Staphylococcus aureus	GyrB	644	Ile 51	Asn 54	Glu 58	Asp 81	**Gly 85**	**Ile 102**	Ser 128	**Arg 144**	Gly 172	Thr 173
S. pneumoniae	GyrB	648	Ile 50	Asn 53	Glu 57	Asp 80	Gly 84	Val 101	**Ser 127**	Lys 143	Gly 171	Thr 172
S. coelicolor	GyrB	687	Val 70	Asn 73	Glu 77	Asp 100	Gly 104	Val 121	Val 147	Thr 163	Gly 191	Thr 192
S. spheroides	**GyrB^R**	677	Leu 63	Asn 66	Glu 70	Asp 93	Gly 97	Val 114	Leu 140	Thr 156	Gly 184	Thr 185
S. rishiriensis	**GyrB^R**	677	Leu 63	Asn 66	Glu 70	Asp 93	Gly 97	Val 114	Leu 140	Thr 156	Gly 184	Thr 185
S. roseochromogenes	**GyrB^R**	677	Leu 63	Asn 66	Glu 70	Asp 93	Gly 97	Val 114	Leu 140	Thr 156	Gly 184	Thr 185
S. coelicolor	ParY	707	Ile 60	Asn 63	Glu 67	Asp 90	Gly 94	Val 111	Ala 137	Leu 153	Gly 197	Thr 198
S. rishiriensis	**ParY^R**	702	Ile 56	Asn 59	Glu 63	Asp 86	Gly 90	Ala 107	Ala 132	Arg 148	Gly 192	Thr 193
S. roseochromogenes	**ParY^R**	702	Ile 56	Asn 59	Glu 63	Asp 86	Gly 90	Ala 107	Ala 132	Arg 148	Gly 193	Thr 194

[a] Resistance proteins of the aminocoumarin producers are shown in bold face. Boldface amino acids are positions of known mutations conferring resistance to aminocoumarins in *E. coli*, *Staphylococcus aureus* (28), or *Streptococcus pneumoniae* (21). The interaction of *E. coli* gyrase with aminocoumarins has been described (16, 32).

the same experimental procedure for resistance determination as described for the *gyrB^R* and *parY^R* genes.

The results are shown in Table 2. The expression of both *novA* from *S. spheroides* and *couR5* from *S. rishiriensis* in *S. lividans* TK24 provided resistance against novobiocin and coumermycin A_1, but the level of resistance was lower than that observed upon expression of *gyrB^R* or *parY^R*. This suggests that *novA* and *couR5* are involved in the transport of novobiocin and coumermycin A_1, presumably in the sequestration of these antibiotics into the medium, but that the principal resistance mechanism of the aminocoumarin antibiotic producers appears to be the synthesis of coumarin-resistant topoisomerases rather than efflux mechanisms.

Ritchie and coworkers (20) reported the cloning of two DNA fragments from the novobiocin producer which conveyed a low level of resistance to novobiocin. However, the restriction map given for these fragments is not in agreement with the sequence of *novA*.

DISCUSSION

The principal resistance mechanism of the novobiocin producer *S. spheroides* has been reported to be the de novo synthesis of an aminocoumarin-resistant GyrB subunit, i.e., GyrB^R, in the presence of novobiocin (30). By cloning of the biosynthetic gene clusters of novobiocin, clorobiocin, and coumermycin A_1, we have now shown that a *gyrB^R* gene is contained in the biosynthetic gene clusters of all three aminocoumarin producers. Unexpectedly, the biosynthetic gene clusters of clorobiocin and coumermycin A_1 were found to contain an additional gene with obvious sequence similarity to genes encoding type II topoisomerase B subunits. This new gene was termed *parY^R*.

Many organisms contain two type II topoisomerases, i.e., gyrase and topoisomerase IV (3, 34). Gyrase is encoded by the genes *gyrA* and *gyrB*, and their gene products form the heterotetramer $(GyrA)_2(GyrB)_2$. Gyrase is unique within the topoisomerases by its ability to introduce negative supercoils into DNA, energetically driven by ATP hydrolysis. Its biological function is to control DNA supercoiling and to relieve topological stress arising during the transcription and replication processes.

Topoisomerase IV is encoded by the genes *parC* and *parE*. Their gene products correspond functionally to GyrA and GyrB, and they also form an active $(ParC)_2(ParE)_2$ heterotetramer. This complex functions as a decatenating enzyme and resolves the interlinked daughter chromosomes following DNA replication. It has been suggested that topoisomerase IV is an important target, in some organisms even the principal target, of the fluoroquinolones and the aminocoumarin antibiotics (7, 9).

However, several organisms do not contain a topoisomerase IV, e.g., *Deinococcus radiodurans*, *Campylobacter jejuni*, and the actinobacteria *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Corynebacterium glutamicum*. In the entire class of actinobacteria, 118 putative *gyrB* genes but no *parE* genes have been identified (www.seasquirt.mbio.co.jp). Expression of the gyrase of *Mycobacterium smegmatis* (17) revealed that this enzyme exhibits, besides supercoiling activity, pronounced decatenating activity and is therefore likely to fulfill both functions in the organism.

Streptomycetes belong to the actinobacteria. The genomic sequence of *Streptomyces coelicolor* A3(2) (1) contains a *gyrA* gene and an adjacent *gyrB* gene (SCO3873 and SCO3874, respectively) and in addition two further genes (SCO5836 and SCO5822) with sequence similarity to the A and B subunits of type II topoisomerases, respectively. These genes are separated by a stretch of 13 kb in the genome and have been annotated in the database as DNA gyrase-like protein, subunit A and probable DNA gyrase subunit B, respectively. Their function has not been investigated so far. We will use the names *parX* (SCO5836) and *parY* (SCO5822) for these genes hereafter. Homologues of *parX* and *parY* apparently exist in the genome of *Streptomyces avermitilis* (http://avermitilis.ls.kitasato-u.ac.jp), although their sequence is not available to the public. These genes (SAV2423 and SAV2442) have been annotated as putative subunits of topoisomerase IV, but no experimental evidence on their function exists.

The deduced *parY* gene product of *S. coelicolor* shows 88% sequence identity with the products of the *parY*^{R} resistance genes of *S. rishiriensis* and *S. roseochromogenes* identified in this study (Fig. 2). Sequence comparison with the *gyrB* and the *parE* genes of the most closely related, high-GC-content, gram-positive bacteria shows that the *parY* genes can be placed neither into the *gyrB* nor into the *parE* group (Fig. 2B). A very similar picture arises from sequence comparison of the *gyrA*, *parC*, and *parX* sequences (Fig. 2A).

Expression of *parY*^{R} from *S. rishiriensis* in *S. lividans* TK24 resulted in novobiocin and coumermycin A_1 resistance, similar to that observed upon expression of *gyrB*^{R}. This suggests that ParY^R is able to function as a coumarin-resistant gyrase B subunit, in clear contrast to ParE of *E. coli*. In *E. coli*, complementation experiments showed that gyrase (consisting of GyrA and GyrB) can partly substitute for topoisomerase IV, but topoisomerase IV (encoded by ParC and ParE) cannot substitute for gyrase, since topoisomerase IV does not possess the unique supercoiling activity of gyrase (3, 24).

The function of the ParY proteins in comparison to topoisomerase IV of other organisms needs further investigation. From the results of our study, it appears possible that streptomycetes may possess two type II topoisomerases, $(GyrA)_2(GyrB)_2$, involved in supercoiling, and $(ParX)_2(ParY)_2$, involved in both supercoiling and decatenation. The coumarin antibiotic producers contain an additional gene coding for a coumarin-resistant GyrB^R protein which can replace the coumarin-sensitive GyrB protein in the A_2B_2 heterotetramer. Likewise, the coumarin-resistant ParY^R protein may replace the coumarin-sensitive ParY protein in the $(ParX)_2(ParY)_2$ heterotetramer in the producers of clorobiocin and coumermycin. The absence of a *parY*^{R} resistance gene in the producer of novobiocin may be related to the lower affinity of novobiocin to topoisomerase IV compared to the affinity of clorobiocin and coumermycin A_1 (12, 22).

Gyrase B mutations which convey resistance to aminocoumarins mostly involve Arg 136 in *E. coli* or corresponding arginine residues in other organisms (5, 6, 32). Arg 136 has been shown to be of principal importance in the binding of aminocoumarin drugs to gyrase (13, 16). However, Arg 136 is not conserved even in the aminocoumarin-sensitive GyrB^S proteins of *S. coelicolor* and *S. spheroides*, or in the ParY protein of *S. coelicolor* (Table 3). On the contrary, only the aminocoumarin-resistant ParY^R proteins of *S. rishiriensis* and *S. roseochromogenes* contain an arginine residue in this position (Table 3). Likewise, nearly all amino acids shown by X-ray studies to be involved in the binding of novobiocin and/or clorobiocin to gyrase were well conserved in the deduced GyrB^R and ParY^R sequences (Table 3). It is therefore not clear why aminocoumarins cannot bind to these enzymes. Computer modeling of the three-dimensional structure of these proteins may help to elucidate the reason.

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