A mutation in the conserved segment of the rpoC gene, which codes for the largest RNA polymerase (RNAP) subunit, β′, was found to make *Escherichia coli* cells resistant to microcin J25 (MccJ25), a bactericidal 21-amino acid peptide active against Gram-negative bacteria (Delgado, M. A., Rintoul, M. R., Farias, R. N., and Salomon, R. A. (2001) J. Bacteriol. 183, 4543–4549). Here, we report that mutant RNAP prepared from MccJ25-resistant cells, but not the wild-type RNAP, is resistant to MccJ25. We also report the isolation of additional rpoC mutations that lead to MccJ25 resistance in vivo and in vitro. The new mutations affect β′ amino acids in evolutionarily conserved segments G, G′, and F and are exposed into the RNAP secondary channel, a narrow opening that connects the enzyme surface with the catalytic center. We also report that previously known rpoB (RNAP β subunit) mutations that lead to streptolydigin resistance cause resistance to MccJ25. We hypothesize that MccJ25 inhibits transcription by binding in RNAP secondary channel and blocking substrate access to the catalytic center.

Mutations of Bacterial RNA Polymerase Leading to Resistance to Microcin J25*

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Bacterial RNA polymerase (RNAP) is the central enzyme of gene expression and a target of genetic regulation. The catalytically proficient core enzyme is composed of five polypeptides: the largest subunit β′, the second largest subunit β, the dimer of identical σ subunits, and a small subunit ω. Upon the binding of one of the several σ specificity subunits the core is converted to a holoenzyme that can specifically initiate transcription from promoters.

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The abbreviations used are RNAP, RNA polymerase; Stl, streptolydigin; MccJ25, microcin J25; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
Microcin J25-resistant Mutants of RNA Polymerase

In the structural model of RNAS core from thermophilic eubacterium Thermus aquaticus a residue equivalent to E. coli β' Thr931 is exposed on the inner surface of RNAP secondary channel, a narrowing that leads from RNAP surface to the catalytic center (24). Based on structural considerations, the secondary channel was hypothesized to direct substrates toward the enzyme active site and to accept the 3'-end-proximal portion of the nascent RNA in transcription elongation complexes that assumed the dead-end conformation (24–26). Thus, the location of the residue affected by rpoC. MccJ25 resistance mutation suggests a novel mechanism of RNAP inhibition: occlusion of RNAP secondary channel. Here, we report the isolation of several MccJ25 resistance mutations in evolutionarily conserved segments G, G', and F of cloned E. coli rpoC. The locations of the corresponding β' residues on the T. aquaticus RNAP structure are exposed in the inside surface of RNAP secondary channel, strongly supporting the idea that MccJ25 inhibits transcription by binding to and occluding this channel.

EXPERIMENTAL PROCEDURES

Bacterial Techniques and DNA Manipulations—Plasmids pRW308 (27) and pRL663 (12), overproducing wild-type or C-terminally hexahistidinyl-subunit, respectively, were used to obtain MccJ25-resistant strains. MccJ25 resistant rpoC mutants generated by error-prone PCR were selected from plasmid banks described by Weibaecher et al. (27). To generate site-specific mutations in segment G, a derivative of pRW308 harboring a unique XhoI site at rpoC codon 943 was created by PCR mutagenesis. The β' subunit encoded by the resultants plasmid, pRW308Xho_943, was wild-type because of the degeneracy of the genetic code. The rpoC positions 928, 929, 930, and 931 were next randomized using mutagenic oligonucleotides complementary to rpoC codons 928–946 and incorporating a XhoI site at codon 943. At the site of randomization, positions corresponding to the first and second bases of the codon were equimolar mixtures of A, G, C, and T, whereas positions corresponding to the third base of the codon was an equimolar mixture of G and C. Mutagenic oligonucleotides were used as primers in a PCR reaction with pRW308Xho_943 template. As a second primer, an oligonucleotide whose sequence corresponded to rpoC positions 2534–2555 was used. This primer anneals upstream of a unique pRW308 SalI site located at rpoC position 2629. After amplification, PCR fragments were treated with SalI and XhoI and ligated into appropriately treated pRW308Xho_943. Ligation mixtures were transformed in MccJ25-sensitive DH5α E. coli host cells, and transformants were plated on solid LB medium containing 200 μg/ml ampicillin. After overnight growth at 37°C, recombinant colonies were replica-plated on LB plates containing 200 μg/ml ampicillin, 50 μg/ml MccJ25 (purified as described previously; see Ref. 20), and 1 mM IPTG to derepress the lac promoter that drives expression of plasmid-borne rpoC. MccJ25-resistant colonies were purified, and plasmid DNA was prepared and retransformed into DH5α E. coli cells. Transformants were plated on plates containing MccJ25 to confirm that resistance is plasmid-borne. An entire SalI-XhoI rpoC fragment was next sequenced at the Rockefeller University DNA technology center to establish the nature of the mutational change leading to MccJ25 resistance.

To randomize rpoC codons 1136 and 1137 (evolutionarily conserved segment G) we made use of a unique SgrAI recognition site at rpoC position 3402 (codon 1134). Mutagenic oligonucleotides spanned the SgrAI sites and two positions to be randomized. The primer used in a primer with pRL663 template and another primer, whose sequence was complementary to rpoC positions 3745–3777. This primer anneals downstream of a unique pRL663 BspEI site located at rpoC position 3639. PCR fragments were treated with SgrAI and BspEI and ligated with appropriately treated pRL663, and MccJ25-resistant clones were selected and confirmed as above. In addition to the SgrAI-BspEI fragment, a portion of rpoC coding for β' segments F and G in mutant plasmids was also sequenced, and no changes from the published sequence were observed. Construction of the β'Δ(943–1130) mutation will be described elsewhere.†

Preparation of Mutant RNA Polymerases and in Vitro Transcription—Highly pure RNAS from MccJ25-resistant E. coli SBG231cells (18) and parental MccJ25-sensitive AB259 cells were purified as described (20). RNAS from Xanthomonas arvense was purified as described in Ref. 31. RNAS from Pseudomonas aeruginosa 8882 strain (provided by Dr. A. Chakrabarty, University of Illinois College of Medicine) was purified by standard E. coli procedure without modifications. Bacillus subtilis RNAS was purified from B. subtilis PolHis cells harboring a genomic rpoC genetically fused to hexahistidine tag (generously provided by Drs. C. P. Moran and G. Schinas, Emory University School of Medicine). RNAS was purified from cell lysates by nickel-nitrilotriacetic acid affinity chromatography followed by ion-exchange on Resource Q (Amersham Biosciences) column. Recombinant RNAS from T. aquaticus was purified by overexpressing E. coli cells as described in Ref. 32. Yeast RNAS II and RNAS III were generous gifts of Dr. Sergei Korolev (SUNY Brooklyn) and George Kassavetis (UCSD), respectively.

Mutant β' Δ(943–1130) RNAS was purified by chitin-affinity chromatography and intein-mediated removal of the chimbin binding domain tag, followed by heparin affinity column chromatography, as described elsewhere. To partially purify RNAS containing β' expressed from a plasmid, E. coli 397C cells (29) were transformed with pRW308, pRL663, or their derivatives, grown at 30°C in 200 ml of LB medium containing 200 μg/ml ampicillin until A660 of 0.5, induced with 1 mM IPTG for 4 h, collected, disrupted by sonication, and polymin P fractionation was performed as described by Kashlev et al. (28). 1 mM NaCl extract of polymin P pellet containing ∼10% pure RNAS was precipitated with ammonium sulfate, and precipitate was stored at −80°C. Before use, an aliquot of ammonium sulfate pellet was dissolved in transcription buffer (40 mM Tris-HCl, pH 7.9, 40 mM KCl, 10 mM MgCl2, 5% glycerol) to give a final protein concentration of ∼1 mg/ml, and this preparation was used in transcription assays.

Transcription from the T7 A1 promoter-containing DNA fragment was performed in 10-μl transcription buffer reactions containing 50 ng of DNA, 0.5 μg of wild-type or mutant RNAS, 0.5 μM Cap primer, 2.5 μM α-32PUTP (300 Ci/mmol), and different concentrations of MccJ25. Reactions proceeded for 10 min at 37°C and were terminated by the addition of urea-containing loading buffer. Products were analyzed by urea-PAGE electrophoresis (7M urea, 20% polyacrylamide), followed by autoradiography and PhosphorImager analysis. Transcription from B. subtilis aegA promoter (32) was performed in a buffer containing 40 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, 25 μg/ml bovine serum albumin using 0.5 mM UpA primer and 2.5 μM α-32PUTP (300 Ci/mmol) substrate.

RESULTS

RNA Polymerase from Microcin-resistant E. coli Cells Is Resistant to MccJ25 in Vitro—Earlier, one of our groups (18) reported that E. coli cells harboring the sjmA1 mutation, but not the wild-type E. coli, were able to grow on selective medium containing MccJ25. The sjmA1 mutation was found to correspond to a substitution of Thr931 to Ile in the largest subunit of E. coli RNAP, the β' subunit. The original report also established that MccJ25 partially inhibited a steady-state in vitro transcription by the wild-type E. coli RNAP, strongly implying that RNAP is a direct target of MccJ25. However, RNAP harboring the T931I substitution was not tested in these experiments. The experiment presented in Fig. 1 demonstrates that the mutant enzyme is indeed resistant to MccJ25 in vitro. As can be seen, MccJ25 inhibited T7 A1 promoter-directed synthesis of the CpaApU abortive RNA product from the Cpa dinucleotide primer and radioactively labeled UTP by the wild-type RNAP (compare lanes 4 and 5). In contrast, the CpaApU synthesis by RNAP purified from cells harboring the sjmA1 mutation was unaffected by the drug (compare lanes 1 and 2). Order-of-addition experiments established that MccJ25 inhibited abortive RNA synthesis when added either before or after the formation of open promoter complex on the T7 A1 promoter-containing DNA fragment used as a template in this experiment (compare lanes 5 and 6). We therefore conclude that (i) RNAP is a true cellular target of MccJ25, and (ii) MccJ25 does not act by preventing RNAP interaction with DNA.

Additional Substitutions in Conserved Segment G of the β' Subunit Lead to MccJ25 Resistance—The genetic context of the
Microcin J25-resistant Mutants of RNA Polymerase

Fig. 1. Transcription inhibition by MccJ25. The indicated E. coli RNAP holoenzymes were combined with the T7 A1 promoter-containing DNA fragment, CpApU primer, and [γ-32P]UTP in the presence and in the absence of 10 μM MccJ25. Reactions were incubated at 37 °C for 15 min, and the products were resolved by denaturing PAGE and revealed by autoradiography. In lanes 2 and 5, MccJ25 was added before promoter complex formation, and in lanes 3 and 6, it was added after promoter complex formation. WT, wild-type.

sjmA1 mutation is shown in Fig. 3. As can be seen, the corresponding substitution occurred in a highly conserved segment of the E. coli β′ subunit, segment G. We hypothesize that the T931I substitution causes MccJ25 resistance by preventing MccJ25 binding to RNAP and that Thr931 is a part of MccJ25 binding site. Given the very high level of evolutionary conservation of segment G, the following two questions are of interest. First, can other MccJ25-resistant mutations in segment G be obtained? Second, will MccJ25 inhibit RNAPs from organisms other than E. coli?

To answer the first question, we obtained plasmids expressing mutant rpoC genes, transformed these plasmids into MccJ25-sensitive E. coli cells, and checked the ability of plasmid-bearing cells to grow on a medium containing MccJ25. In case when growth on selective medium was observed, we purified RNAPs containing mutant β′ and confirmed that mutant RNAPs were indeed resistant to MccJ25. In cases when no in vivo resistance was observed, we considered the possibility that RNAP containing mutant β′ could not support cell growth in the presence of MccJ25, when the wild-type, chromosomally encoded RNAP was inactivated. Therefore, RNAPs containing plasmid-borne β′ were also purified, and their sensitivity to MccJ25 was tested in vitro. All mutants reported below were tested this way. Fig. 2 shows the results of in vivo and complementary in vitro testing with some of the mutants as an example.

A set of several point mutations in segment G of E. coli rpoC cloned on an expression plasmid was recovered in two unrelated screens, one aimed at obtaining termination-altering rpoC mutants (27) and another site-specifically mutating evolutionarily conserved β′ positions 921 and 935,2 is presented atop of the sequence alignment shown in Fig. 3. MccJ25-sensitive E. coli cells were transformed with plasmids expressing mutant rpoC genes, and the ability of plasmid-bearing cells to grow on MccJ25-containing medium was investigated. As controls, cells harboring plasmids expressing wild-type rpoC or MccJ25-resistant rpoCT931I allele were employed. As expected, cells expressing wild-type rpoC were sensitive to MccJ25, whereas cells expressing the T931I allele were resistant (Fig. 2 and data not shown). Cells harboring expression plasmids bearing the F935S substitution were as resistant as control cells expressing rpoCT931I, whereas cells expressing the R933H,A946V double mutant resulted in slow but detectable growth on MccJ25-containing medium (Fig. 2 and data not shown). In contrast, cells expressing Q921P, T934M, and H936Y alleles did not grow on selective medium (Fig. 2 and data not shown).

The results of in vitro transcription assays correlated with the in vivo results (Fig. 2) (data not shown). However, the R933H,A946V double mutant, which showed low levels of resistance in vivo, was highly resistant in vitro, suggesting that the mutant RNAP in vivo function is impaired. RNAP harboring the F935S substitution was found to be resistant to the drug, whereas other mutants were sensitive. Three RNAP harboring dominant lethal mutations in segment G, M932L, R933S, and T934A, were also tested for MccJ25 resistance. These mutants were obtained in the course of an independent mutagenesis effort3 and were prepared by in vitro reconstitution.

Additional MccJ25-resistant mutants in segment G were also sought directly. Three rpoC codons immediately to the left of position 931 (928, 929, and 930) were randomized by site-directed PCR mutagenesis, libraries of recombinant plasmids were transformed in MccJ25-sensitive E. coli cells, and MccJ25-resistant clones were selected. As a control, position 931, the site of the original MccJ25-resistant mutation, was also randomized. MccJ25-resistant clones were only obtained in the control mutagenesis reaction. Sequencing of three resistant clones revealed the presence of the original mutation, T931I, as well as two new mutations, T931N and T931L. The corresponding enzymes were also resistant in vitro (data not shown). The result thus suggests that the identity of β′ amino acids 928–930 is either not important for MccJ25 inhibition, or MccJ25-resistant substitutions at these positions lead to lethal phenotype.

MccJ25 Effect on RNAPs Other Than E. coli—MccJ25 is effective against Gram-negative bacteria but has no effect on Gram-positive bacteria (19). To determine the specificity of transcription inhibition by MccJ25, we assembled a panel of RNAPs prepared from several Gram-negative and Gram-positive bacteria and compared their ability to perform abortive RNA synthesis in the presence or in the absence of MccJ25 (Fig. 4). In the absence of MccJ25, RNAPs from Gram-negative bacteria demonstrated approximately equal specific activities on the T7 A1 promoter (0.9, 0.6, 1.2, and 0.8 pmol/min of CpApU synthesized by 1 pmol of wild-type E. coli RNAP, E. coli RNAP transposon, P. aeruginosa RNAP, and X. oryzae RNAP, respectively). In agreement with the previously determined in vivo specificity, MccJ25 inhibited abortive synthesis of CpApU from the T7 A1 promoter-containing DNA fragment by RNAPs prepared from three Gram-negative bacteria, wild-type E. coli, X. oryzae, and P. aeruginosa (see Fig. 4; 10, 11, and 9% residual activity in the presence of 25 μM MccJ25, respectively). As expected, E. coli RNAP transposon was active in the presence of 25 μM MccJ25 (85% activity). RNAP from T. aquaticus was assayed on the T7 A1 promoter at 60 °C and was considerably less active (0.2 pmol of CpApU synthesized per min per pmol of enzyme). MccJ25 had no effect on abortive synthesis by recombinant T. aquaticus RNAP at 60 °C (see Fig. 4; 100% activity in the presence of 25 μM MccJ25).

Because RNAP from B. subtilis displays only a very low level of activity on the T7 A1 promoter (data not shown), we assayed the effect of MccJ25 on this enzyme during the abortive synthesis of UpApG on B. subtilis vegA promoter (32). In the absence of MccJ25, B. subtilis vegA promoter, E. coli RNAP, and E. coli RNAP transposon demonstrated comparable levels of activity on the vegA promoter (0.8, 0.3, and 0.1 pmol of UpApG synthesized per min per pmol of RNAP, respectively). MccJ25 had no effect on the B. subtilis enzyme (see Fig. 4; 110% activity in the presence of 25 μM MccJ25) and E. coli RNAP (see Fig. 4; 85% activity in the presence of 25 μM MccJ25) but was active against wild-type E. coli enzyme on this promoter (see Fig. 4; 11% activity in the presence of 25 μM MccJ25). Additional experiments demonstrated that MccJ25 had no effect on transcription by yeast RNAPs II and III (data not shown).

3 V. Epshtein, A. Mustaev, and A. Goldfarb, submitted for publication.
Effect of Substitutions in and around Conserved Region G on MccJ25 Resistance—Because segment G positions affected by MccJ25-resistant substitutions are identical in H9252/H11032 homologues from Gram-positive and Gram-negative organisms, the result implies that other regions of RNAP may also contribute to MccJ25 binding. In RNAP from Gram-negative bacteria, segment G is followed by a long stretch of amino acid sequence that is hypervariable in evolution (33). The hypervariable region is missing in RNAPs from Gram-positive bacteria and eukaryal RNAPs. To test whether the presence of the evolutionarily hypervariable region of β' contributes to the MccJ25 sensitivity of RNAP from Gram-negative bacteria, we tested the ability of rpoC/H9004 (943–1130) allele that lacks the entire hypervariable region and thus resembles homologues from Gram-positive microorganisms to confer MccJ25 resistance in vivo. The mutant poorly assembles into RNAP, presumably because of its inability to compete with chromosomally encoded wild-type β'. We therefore tested the ability of MccJ25-sensitive cells harboring plasmid pIA331, which, in the presence of IPTG, co-overexpresses wild-type rpoA (α), rpoB (β), and rpoC (943–1130) and thus increases the efficiency of the mutant enzyme assembly to grow on MccJ25-containing medium. As controls, plasmid pIA423, which co-overexpresses wild-type E. coli RNAP, and pRL663rpoCT931I plasmids, were used. As can be seen from Fig. 5A, cells harboring plasmid pIA331 and pRL663rpoCT931I, but not cells harboring pIA423 and pRL663, formed colonies in the presence of MccJ25 and IPTG. Colonies formed by cells harboring plasmid pIA331 were minute as compared with colonies formed by cells harboring pRL663rpoCT931I cells, but the efficiency of plating was comparable. Plasmid pIA331, but not other plasmids, significantly inhibited cell growth in the presence of IPTG only, suggesting that RNAP (943–1130) was defective in some cellular function(s) unrelated to MccJ25 resistance. Be that as it may, the results demonstrate that hypervariable region indeed contributes to MccJ25 sensitivity and may be partially dispensable for cell viability at our conditions, because RNAP lacking β' residues 943–1130 is presumably the only transcriptionally active enzyme in the presence of MccJ25.

E. coli RNAP (943–1130) was prepared from cells harboring pIA331 and tested for the ability to transcribe from the T7 A1 promoter in the presence or in the absence of MccJ25 (Fig. 5B). The results showed that the mutant was more resistant to the drug than wild-type E. coli RNAP (26 and 3% residual activity in the presence of 50 μM MccJ25). Because RNAPs from Gram-positive bacteria are highly resistant to MccJ25 in vitro, other

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4 I. Artsimovitch, personal observation.
Halobacterium halobium (Taq), /H11032 and G in vivo, P. aeruginosa MccJ25 resistance (either in vivo sites of natural splits in homologues from chloroplasts and archaea. Hatched boxes UpApG product. WT in the form a continuous stretch of evolutionary conserved sequence homologues from Gram-positive bacteria, segments G and G/H11032 ment G variable segment is followed by evolutionarily conserved seg-

sites in these RNAPs must contribute to MccJ25 resistance. In the β’ subunits from Gram-negative bacteria, the hypervariable segment is followed by evolutionarily conserved segment G’ (33). Because there is no hypervariable region in homologues from Gram-positive bacteria, segments G and G’ form a continuous stretch of evolutionary conserved sequence in the β’ subunits from these organisms (Fig. 3). Segment G residues that cause MccJ25 resistance are part of the so-called G-loop in RNAP structures from thermophilic bacteria of the Thermus genus (24, 34, 35). Residues of segment G’ are also part of the G-loop. In particular, Thermus RNAP residues corresponding to E. coli β’ amino acids 1137 and 1138 are in direct contact with the residue corresponding to E. coli Thr303 and are located at the base of the G-loop. We therefore considered a possibility that substitutions in positions 1137 and 1138 will make E. coli RNAP MccJ25-resistant. Accordingly, codons 1137 and 1138 of plasmid-borne rpoC were randomized, mutant plasmid libraries were transformed in MccJ25-sensitive E. coli, and transformants were plated on selective medium containing MccJ25. No MccJ25-resistant mutants were obtained when codon 1137 was randomized. One clone was picked up at random and found to encode a G1137A substitution; the corresponding RNAP was MccJ25-sensitive in vitro. One resistant clone, coding for L1138T, was recovered from codon 1138 mutagenesis. The corresponding RNAP was purified and found to be MccJ25-resistant in vitro (Fig. 2). Because no changes from the published rpoC sequence in segment G was observed in this mutant (data not shown), we conclude that a substitution in segment G’ is indeed responsible for MccJ25 resistance. One MccJ25-sensitive clone from the 1138 mutagenesis reaction was picked up at random and sequenced and found to contain a mutation coding for E1030K substitution; the corresponding RNAP was MccJ25-sensitive in vitro. The corresponding RNAP was purified and found to be MccJ25-resistant in vitro (Fig. 2). Because no changes from the published rpoC sequence in segment G was observed in this mutant (data not shown), we conclude that a substitution in segment G’ is indeed responsible for MccJ25 resistance. One MccJ25-sensitive clone from the 1138 mutagenesis reaction was picked up at random and sequenced and found to contain a mutation coding for E1137A substitution; the corresponding RNAP was MccJ25-sensitive in vitro. One resistant clone, coding for L1138T, was recovered from codon 1138 mutagenesis. The corresponding RNAP was purified and found to be MccJ25-resistant in vitro (Fig. 2). Because no changes from the published rpoC sequence in segment G was observed in this mutant (data not shown), we conclude that a substitution in segment G’ is indeed responsible for MccJ25 resistance. One MccJ25-sensitive clone from the 1138 mutagenesis reaction was picked up at random and sequenced and found to contain a mutation coding for E1137A substitution; the corresponding RNAP was MccJ25-sensitive in vitro. One resistant clone, coding for L1138T, was recovered from codon 1138 mutagenesis. The corresponding RNAP was purified and found to be MccJ25-sensitive in vitro (data not shown).

A functional deletion of β’ amino acids 1145–1198 immediately to the right of segment G’ was described by us previously (33). This deletion did not result in MccJ25 resistance in vivo and in vitro (data not shown).

A double mutation coding for E1030K and I1134D substitutions was isolated in an independent PCR-based screen for termination-altering rpoC mutations (27). The first substitution, of Glu1030, occurred in the hypervariable region; the second substitution, of Ile1134, occurred in segment G’. We tested the ability of plasmid-borne E1030K, I1134D allele to confer MccJ25 resistance in vivo and in vitro and observed no resist-
ance (data not shown). We also looked for additional MccJ25-resistant mutations within the bank of rpoC expression plasmids subjected to error-prone PCR at and around segments G and G'/H11032 (rpoC codons 876–1213; see Ref. 27). A triple mutation coding for I1115V, G1136D, and F1145S substitutions was recovered in this way. Of the three residues affected, one (G9252/H11032 Phe1145) is removed by MccJ25-sensitive (1145–1198) deletion. Substitutions I1115V and/or G1136D are thus likely responsible for MccJ25 resistance. Because I1115V is a conservative substitution, substitution of evolutionarily conserved Gly1136 in segment G'/H11032 is the probable cause of MccJ25 resistance.

Substitutions in Evolutionarily Conserved Segment F Lead to MccJ25 Resistance—Residues of G/H9252/H11032 segments G and G'/H11032 that are important for MccJ25 inhibition are exposed on the surface of narrow RNAP secondary channel that opens on the downstream face of the enzyme and leads to the catalytic site (24). In addition to G/H9252 segments G and G', conserved segment F also participates in the formation of the secondary channel. We were therefore interested in whether MccJ25-resistant mutations in segment F can be obtained. Toward this end, we tested two segment F mutants, F773I and S793F, that were shown previously to cause resistance to the elongation inhibitor, streptolydigin (12). These mutants did not result in appreciable MccJ25 resistance in vivo or in vitro (Fig. 2) (data not shown). We therefore looked for MccJ25-resistant region F mutants directly, by incorporating an error-prone PCR-amplified rpoC fragment coding for region F (rpoC codons 544–875) into an rpoC expression plasmid, transforming mutant plasmids in MccJ25-sensitive host, and selecting MccJ25-resistant colonies. Several independent MccJ25-resistant colonies were obtained, and the plasmid-borne nature of MccJ25 resistance was confirmed by retransforming of rpoC expression plasmids from MccJ25-resistant clones into sensitive host and replating on selective medium. Four independent clones were obtained, and their sequence at and around segments F, G, and G'/H11032 was determined. No changes in segment G'/H11032 sequences was detected. In contrast, changes from the published sequence leading to substitutions of segment F residues Ser733 for Pro, Leu783 for Gln, and a double substitution of Leu746 for Pro and...
Phe773 for Ile were observed (Fig. 6). In vitro analysis confirmed that RNAPs carrying mutations in segment F are resistant to MccJ25 (Fig. 2) (data not shown). We conclude that substitutions in RNAP segment F lead to MccJ25 resistance.

In the case of stl-resistant mutations, the main cluster of stl-resistant mutations is located in the E. coli subunit, between Rif clusters I and II (3). To further investigate the relationship between MccJ25 and Stl resistance, we tested the ability of E. coli cells expressing several MccJ25-resistant alleles to grow in the presence of Stl. We observed that cells overproducing Stl-resistant rpoB alleles formed minute colonies in the presence of stl, whereas cells overproducing Stl-sensitive rpoB did not grow (data not shown). None of the segment F, segment G, or segment G’ MccJ25-resistant rpoC mutations tested allowed growth on Stl-containing plates (data not shown).

Plating of cells expressing Stl-resistant rpoB alleles on MccJ25 gave an unexpected result. As expected, cells expressing rpoC793F grew in the presence of MccJ25, whereas cells expressing wild-type rpoC did not grow (data not shown). None of the segment F, segment G, or segment G’ MccJ25-resistant rpoC mutations tested allowed growth on Stl-containing plates (data not shown).

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picin-resistant rpoB mutants and found that none of them were able to support growth in the presence of MccJ25 (data not shown). We conclude that Stl-resistant mutations in the rpoB gene lead to MccJ25 resistance.

**CONCLUSIONS**

The principal result of this work is the demonstration that transcription by mutant RNAP purified from MccJ25-resistant E. coli cells is resistant to MccJ25, whereas transcription by RNAP purified from wild-type cells is MccJ25-sensitive. This result proves that E. coli RNAP is the cellular target of MccJ25. In the best understood case of Rifampicin, mutations toward resistance affect RNAP residues that are removed from each other in primary sequence but that cluster in the enzyme quaternary structure (1–3, 24). Structural analysis demonstrates that Rif-resistance mutation define the Rifampicin binding site (4). We hypothesized that the original MccJ25-resistance mutation, that changed evolutionarily conserved amino acid inside RNAP secondary channel, likewise defines the MccJ25 binding site on RNAP. According to this view, MccJ25 inhibits transcription by binding in the secondary channel and preventing the traffic of NTP substrates to the catalytic center of the enzyme. Indeed, molecular modeling using T. aquaticus RNAP structure and a reported MccJ25 structure (20) shows that 21-amino acid MccJ25 can fit into RNAP secondary channel with little or no steric clashes (data not shown).

If MccJ25 were indeed binding in the secondary channel, it should be possible to isolate additional MccJ25-resistant mutations located in the channel. Further, at least some of these residues must be evolutionarily variable, to explain the observed restriction of MccJ25 action to RNAP from Gram-negative bacteria. Both of these predictions are fulfilled. Here, we report the isolation of additional MccJ25-resistant mutants in segment G, as well as mutations in conserved segments G’ and F. Structural analysis indicates that in T. aquaticus RNAP core enzyme structure, residues homologous to those affected in E. coli can be mutated toward Stl resistance mutation is colored red. The view on the right is perpendicular to the main DNA binding channel of the enzyme and was obtained from the left view by ~90° clockwise rotation around the vertical axis.
each other and are located on two opposing sides of the secondary channel, as well as on the roof of the channel. Residues in segments G and G’ are identical in RNAPs from Gram-positive and Gram-negative bacteria and therefore could not be responsible for differential action of MccJ25 on these enzymes. On the other hand, residues in segment F are different between RNAPs from Gram-negative and Gram-positive bacteria, and these differences could account for observed specificity of MccJ25 inhibition. In addition, our data indicate that the presence of the G-loop is in an “open” conformation and takes part in the formation of the secondary channel wall (Fig. 8, cyan). In the holoenzyme structure, G loop is in an “open” conformation, turned almost 90 degrees from its position in the core. The opening of the G loop shortens the secondary channel and may affect MccJ25 binding. It is conceivable that the hypervariable region restricts the mobility of the G-loop and thus allows better binding of MccJ25 to RNAP from Gram-negative bacteria. The hypervariable region is inserted in RNAP G loop, which appears to be flexible. In _T. aquaticus_ core enzyme structure, G loop is in a “closed” conformation and takes part in the formation of the secondary channel wall (Fig. 8, cyan). In the holoenzyme structure, G loop is in an “open” conformation, turned almost 90 degrees from its position in the core. The opening of the G loop shortens the secondary channel and may affect MccJ25 binding. It is conceivable that the hypervariable region restricts the mobility of the G-loop and thus allows better binding of MccJ25 to RNAP from Gram-negative bacteria.

Earlier (12), we proposed that the presence of mutations that cause resistance to Stl and α-aminitin in conserved segment F of bacterial RNAP β′ subunits and eukaryal RNAP II largest subunits indicated that the two drugs may function similarly in their respective systems despite the lack of common chemical structure. Here, we show that mutations in β′ segment F also cause resistance to MccJ25. Amino acids that, when mutated, cause resistance to all three drugs are exposed on the surface of the secondary channel. Analysis of a structural model of bacterial RNAP elongation complex reveals that RNAP secondary channel provides the only unobstructed way from the solvent to RNAP catalytic center, because access from the main DNA binding channel is blocked by nucleic acids (25). Therefore, it is possible that substitutions in the secondary channel can cause resistance to transcription elongation inhibitors whose actual mechanisms of action are different, but all of whom have to pass through the secondary channel to get access to the catalytic center.

Analysis of Stl-resistant _rpoB_ revealed, unexpectedly, that they cause MccJ25 resistance. The result appears to strengthen the idea that MccJ25 and Stl may have a common inhibition mechanism, despite the lack of structural similarity. On the other hand, the presence of MccJ25-resistant mutations in β is difficult to reconcile with the notion of MccJ25 binding in the secondary channel, because in RNAP structure, the site of Stl-resistant mutations in the β subunit (Fig. 8, red) is located slightly upstream of the catalytic center and should become inaccessible from the secondary channel in the elongation complex (25). Therefore, it is possible that lesions in this site cause MccJ25 resistance indirectly. For example, the G-loop, when opened, may interact with the site of Stl resistance mutations in β, and Stl resistance mutations could therefore affect the position of the G-loop and thus cause resistance to MccJ25. Alternatively, the β Stl site can undergo a conformational change upon transcription complex formation that brings it closer to the secondary channel.

Obviously, further studies will be necessary to determine the site of MccJ25 interaction with RNAP, the mechanism of transcription inhibition by MccJ25, and its relationship, if any, to transcription inhibition by Stl. If MccJ25 were indeed binding to RNAP, several very specific predictions concerning the biochemical effects of its interactions with transcription complex could be made. The secondary channel is thought to conduct NTP substrates to the RNAP catalytic center, to accept the 3′′−end proximal portion of the nascent RNA in the back-tracked, dead-end conformation of the elongation complex, and to accept transcript cleavage factors GreA and GreB (24). MccJ25 binding should interfere with all of these activities. Experiments aimed at testing these predictions are currently underway.

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