Communication

Streptolydigin-resistant Mutants in an Evolutionarily Conserved Region of the β'-Subunit of Escherichia coli RNA Polymerase*

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Mutations conferring streptolydigin resistance onto Escherichia coli RNA polymerase have been found exclusively in the β subunit (Heisler, L. M., Suzuki, H., Landick, R., and Gross, C. A. (1993) J. Biol. Chem. 268, 25369–25375). We report here the isolation of a streptolydigin-resistant mutation in the E. coli rpoC gene, encoding the β'-subunit. The mutation is the Phe793 → Ser substitution, which occurred in an evolutionarily conserved segment of the β'-subunit. The homologous segment in the eukaryotic RNA polymerase II largest subunit harbors mutations conferring α-amanitin resistance. Both streptolydigin and α-amanitin are inhibitors of transcription elongation. Thus, the two antibiotics may inhibit transcription in their respective systems by a similar mechanism, despite their very different chemical nature.

Streptolydigin (Stl)1 is a 3-acyltetramic acid antibiotic (1), which specifically inhibits bacterial DNA-dependent RNA polymerase (2–6). Stl interacts with RNA polymerase in ternary transcription complexes and inhibits growth of nascent RNA chains during transcription initiation and elongation (4, 6). The binding of RNA polymerase to template DNA is not affected by Stl (2). Thus, the likely target of Stl is either the binding of incoming NTP to the substrate binding site of RNA polymerase or the catalysis of phosphodiester bond formation (4, 6).

Transcription by RNA polymerase purified from mutant cells that acquired resistance to the drug is resistant to Stl (3). In Escherichia coli, all Stl-resistant RNA polymerases studied to date have an altered β subunit (7, 8), and all known mutations leading to Stl resistance map to the rpoB gene, which codes for the β subunit (8). Substitutions of amino acids in β between 540 and 546 lead to Stl resistance (9–11). The highest resistance levels in vivo and in vitro were found in the case of substitutions at positions 544 and 545 (10–11). In the absence of more direct data it has been assumed that these β amino acids participate in Stl binding to RNA polymerase.

Since Stl inhibits elongation of nascent RNA, mutations changing RNA polymerase Stl-binding site are likely to change the catalytic properties of the enzyme. However, RNA polymerase with the known β subunit Stl mutations has unaltered transcription elongation and transcription termination properties in vivo and in vitro (11). Moreover, the site of Stl resistance in the β subunit is dispensable for RNA polymerase function, since mutant RNA polymerases with deletions spanning amino acids 534–545 are functional both in vivo and in vitro (10). This apparent discrepancy led Heisler et al. (11) to hypothesize that other site(s) in RNA polymerase may be involved in Stl binding. This hypothesis was substantiated by a report (12) that in RNA polymerase reconstituted in vitro, the β'-subunit from a mutant strain of Bacillus subtilis was responsible for Stl resistance. However, no β'-subunit Stl-resistant mutants have ever been reported in E. coli. Here, we report an isolation and localization of such a mutation. The mutation leads to an amino acid substitution in an evolutionarily conserved region of the β'-subunit. In the β'-homologues from eukaryotic RNA polymerase II, this region harbors mutations that lead to resistance to α-amanitin, a peptide toxin that specifically inhibits RNA chain elongation by RNA polymerase II. Our results raise the possibility that the structurally different inhibitors streptolydigin and α-amanitin may interact and inhibit RNA polymerase from prokaryotic and eukaryotic systems by a similar mechanism.

EXPERIMENTAL PROCEDURES

Bacterial Techniques and DNA Manipulations—Streptolydigin was purchased from BioMol. Cells were grown in LB medium. Plates containing linear gradient of Stl concentration were prepared as described (10). To deprec the lac promoter of the pMK201 plasmid 1 mM isopropyl-β-D-galactopyranoside was included in the selective medium. In vitro hydroxylamine mutagenesis of the pMK201 plasmid was carried out exactly as described by Heisler et al. (11). Standard laboratory techniques were used for subcloning and sequencing of rpoC fragments.

Preparation of Mutant RNA Polymerases and in Vitro Transcription—To purify histidine-tagged RNA polymerase from induced cells containing pMK201 or its derivatives, the procedure described by Kashlev et al. (13) employing affinity chromatography on Ni2+-NTA-agarose (Qiagen) was used.

Transcription of bacteriophage T2 DNA was performed in 100-μl reactions containing 10 μg of T2 DNA, 2 μg of WT or mutant RNA polymerase, 0.5 mM ATP, CTP, and GTP, 0.025 mM [3H]UTP (43 Ci/mM), 10 mM Tris-HCl (pH 7.9), 100 mM NaCl, 50 mM KCl, 10 mM MgCl2, 0.5 mg/ml bovine serum albumin, and different concentrations of Stl. Reactions were initiated by addition of NTPs and proceeded for 15 min at 37°C. Reactions were terminated by addition of 1 ml of 10% trichloroacetic acid, and the amount of acid-insoluble radioactivity was determined.

To determine the elongation rate of the mutant RNA polymerase,
The purpose of this study was to generate mutations in E. coli rpoC gene that would result in StI R phenotype. E. coli cells are naturally resistant to high concentration of StI, due to a per- 
amatinin resistance are indicated. Other \( \beta' \) features, including a surface-exposed putative zinc finger (32), a site where homologues from chloroplasts and archaebacteria are split (33, 34), a site where 9 nucleotide sequence around codon 793 is shown by the open box.

**RESULTS**

The purpose of this study was to generate mutations in E. coli rpoC gene that would result in StI R phenotype. E. coli cells are naturally resistant to high concentration of StI, due to a permutation barrier (2). Mutant E. coli that become sensitive to low concentration of StI can be selected for use of mutant strains with StI-resistant RNAP (2, 11). Throughout this work, we used the StI-sensitive E. coli strain CAG 14064, provided by C. Gross. To obtain StI R mutations, rpoC expression plasmid pMA201 (13) was mutagenized with hydroxylamine in vitro (11). After mutagenesis, plasmid DNA was introduced into CAG 14064 strain by electroporation and cells were plated on media containing 12.5 \( \mu \)g/ml StI. Out of \( \sim 1 \times 10^8 \) plasmid-bearing cells plated on the selective media, 20 StI R clones were obtained. Plasmid DNA was prepared from the resistant clones and retransformed in CAG 14064, and cells were plated on StI-containing media. In this way two pMA201 derivatives (pMA201-15 and pMA201-16) that conferred StI resistance to CAG 14064 strain were selected and used for further analysis. To localize StI R mutations, a series of in vitro exchanges of DNA fragments between the mutant plasmids pMA201-15 and pMA201-16, and the parental pMA201 were performed. The recombinant plasmids were checked for their ability to confer StI resistance to CAG 14064 strain and will refer to the mutation by that name.

**Fig. 1. rpoC mutation S793F.** A, localization of rpoCS793F by transfer of the indicated fragments from plasmid pMK201-15 to recipient parental plasmid pMK201. Restriction sites used are indicated. The failure of recombinant plasmids to confer StI resistance upon the sensitive host Escherichia coli rpoC gene was taken as evidence that the transformed fragmen did not carry the StI R mutation. B, genetic context of rpoCS793F. The heavy bar represents the 1407 amino acid \( \beta' \) subunit of E. coli RNAP. Hatched boxes labeled A–H represent segments of \( \beta' \) highly conserved in evolution. The amino acid sequence of E. coli \( \beta' \) subunit expressed from the mutant plasmids has a phenylala-
The results of plating of CAG 14064 cells expressing rpoCS793F from the pMKa201 plasmid on a plate containing a linear gradient of Stl are shown in Fig. 2A. Cells overproducing the mutant $\beta'$ subunit continued to grow at $\sim$20 $\mu$g/ml Stl, while cells overproducing wild-type $\beta'$ from the pMKa201 failed completely to form colonies on the gradient plate.

The $\beta'$ subunit expressed from the plasmid pMKa201 or its derivatives is extended with a stretch of six consecutive histidine residues at its C terminus (the His tag). As is shown elsewhere, the His-tagged RNAP is indistinguishable from the wild-type RNAP in functional tests and can be easily separated from RNAP with chromosome-encoded $\beta'$ by affinity chromatography on Ni$^{2+}$ sorbent (13). We purified His-tagged RNAP from cells harboring pMK201 or pMK201-rpoCS793F. The response of the two enzymes to Stl was compared in bacterial T2 DNA transcription assay (Fig. 2B). The enzymes displayed equal levels of activity in the absence of Stl (data not shown). In the presence of Stl, the mutant enzyme was clearly more active than the control WT enzyme (half-inhibition at 100 and 10 $\mu$g/ml Stl, respectively).

Recently, one of us performed a systematic search for termination-altering mutations in the cloned E. coli rpoC gene (15). The mutations clustered in several regions of the gene. Many of the termination-altering mutations resulted in amino acid substitutions in a segment of the $\beta'$ subunit between amino acids 630 and 800 (interval 3, see Ref. 15 for nomenclature). Since rpoCS793F is contained within interval 3, we investigated the ability of interval 3 termination-altering mutations to confer Stl$^R$ phenotype to Stl-sensitive cells. CAG 14064 cells harboring 19 pRW308 rpoC expression plasmid derivatives carrying interval 3 mutations were streaked on plates with a linear gradient of Stl. As a control, 8 interval 2 (amino acids 310–390) and 10 interval 5 (amino acids 1305–1370) mutant plasmids were used. Out of 37 plasmids tested, only 5 plasmids with interval 3 mutations (rpoC3302 (M747I), rpoC3309 (R780H), rpoC3310 (G729D), rpoC3312 (E765K), and rpoC3329 (M725I)) conferred very low levels of Stl resistance to CAG 14064 cells (Fig. 3A, and data not shown). rpoC alleles 3302 (M747I) and 3309 (R780H), which conferred higher levels of resistance, were recloned in the pMKa201 plasmid; the two His-tagged mutant RNAPs were purified, and their response to Stl was investigated in the T2 DNA transcription system (Fig. 3B). In the absence of Stl, the mutant enzymes were 50% more active than the control enzyme (data not shown). The two enzymes reproducibly demonstrated slightly higher levels of Stl resistance than the control enzyme (half-inhibition at 25 $\mu$g/ml Stl).

Since Stl inhibits phosphodiester bond formation, it is expected that mutations in Stl-binding site will change the catalytic properties of RNAP. Transcription elongation, transcription pausing, and transcription termination by the three mutant enzymes (M747I, R780H, and S793F) and WT RNAP were investigated in the experiment presented in Fig. 4. The three mutant enzymes elongated RNA at different rates (Fig. 4A); S793F RNAP was slightly “slower” than the WT enzyme, while M747I and R780H enzymes were considerably “faster,” in agreement with the previous data (15). All three mutant enzymes and the WT control demonstrated essentially the same pausing pattern in this assay. Changes in transcription elongation rates were accompanied by changes in transcription termination efficiencies of the mutant enzymes on a factor-independent $\lambda$ tr2 terminator (Fig. 4B); S793F RNAP terminated slightly more efficiently (10% read-through), while M747I and R780H enzymes terminated considerably less efficiently (53 and 48% read-through, respectively) than the wild-type enzyme (20% read-through).

The response of the mutant enzymes to other transcription inhibitors also was investigated. The mutant enzymes were as sensitive as initiation inhibitor rifampicin and elongation inhibitor tagetitoxin as the wild-type control (data not shown).

**CONCLUSIONS**

The principal result of this work is the demonstration that mutations in the $\beta'$ subunit of E. coli RNAP can confer resistance to Stl. From the point of practical E. coli RNAP genetics, the availability of an Stl resistance marker in rpoC should facilitate isolation of loss-of-function rpoC mutations, similarly to the approach that was used with rpb8 (RNAP $\beta$ subunit) mutations employing the rifampicin resistance marker (16, 17).

Biochemical analysis of the three mutant RNAPs (M747I, R780H, and S793F) demonstrates that the extent of defects in transcription elongation, transcription pausing, and transcription termination of the mutants studied is not correlated with the levels of Stl resistance. This situation is reminiscent of that for the $\beta$ subunit Stl$^R$ mutants, which do not demonstrate significant transcription defects (11). We note that the S793F mutation occurred in the highly conserved Segment F of $\beta'$ (Fig. 1B), and that Stl$^R$ mutations affecting RNAP basic function may have escaped our screen which requires mutant RNAP in vivo function.
It is conceivable that additional changes close to E. coli β'-position 793 will be identified that will lead to higher levels of streptolydigin resistance. While this work was in progress, the B. subtilis Stlβ strain (22) was sequenced (40). The B. subtilis mutator projects on E. coli β'-position 792, i.e., just next to Stlβ mutation isolated in this study. Thus, the β'-determinants of RNAP streptolydigin resistance coincide in Gram-negative E. coli and Gram-positive B. subtilis. Bacterial RNAP β'-subunits are highly homologous to the large subunit of eukaryotic RNA polymerase II (18). As is shown in Fig. 1B, the β'-Stlβ mutations characterized in this work occurred in a segment of β' that is highly conserved in evolution (Segment F, according to Ref. 15 nomenclature). Several lines of evidence suggest that this segment plays an important role conserved in all RNA polymerases. Mutations in Segment F that dramatically change nascent RNA elongation rate and/or termination efficiencies were reported in eukaryotic as well as prokaryotic RNAPs (19, 15). A recent cross-linking study demonstrates that the E. coli β'-segment between amino acids 748 and 814, containing most of segment F, is in tight contact with the 3' end of the nascent RNA (23). Finally, in eukaryotic RNA polymerase II largest subunit, Segment F harbors mutations that render RNA polymerase resistant to the elongation inhibitor α-amanitin (Fig. 1B). The discovery of Stlβ mutations in Segment F suggests that despite the lack of structural similarity, Stl and α-amanitin may inhibit transcription by a similar mechanism. Although speculative, this hypothesis is consistent with available biochemical data on the mechanism of Stl and α-amanitin inhibition of transcription. (i) Both are elongation inhibitors, but allow several phosphodiester bonds to be made and different complexes are inhibited to a different extent (4, 20, 21); (ii) both inhibit pyrophosphorylation (3, 4) (2); (iii) both inhibit nascent RNA cleavage by transcription elongation factors (22, 23).4

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