Streptolydigin Resistance Can Be Conferred by Alterations to Either the β or β’ Subunits of Bacillus subtilis RNA Polymerase*

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Rifampicin and streptolydigin are antibiotics which inhibit prokaryotic RNA polymerase at the initiation and elongation steps, respectively. In Escherichia coli, resistance to each antibiotic results from alterations in the β subunit of the core enzyme. However, in Bacillus subtilis, reconstitution studies found rifampicin resistance (RifR) associated with the β subunit and streptolydigin resistance (StlR) with β’. To understand the basis of bacterial StlR, we isolated the B. subtilis rpoC gene, which encodes a 1,199-residue product that is 53% identical to E. coli β’. Two spontaneous StlR mutants carried the same D796G substitution in rpoC, and this substitution alone was sufficient to confer StlR in vivo. D796 falls within Region F, which is conserved among the largest subunits of prokaryotic and eukaryotic RNA polymerases. Among eukaryotes, alterations in Region F promote resistance to α-amanitin, a toxin which inhibits transcription elongation; among prokaryotes, alterations in Region F cause aberrant termination. To determine whether alterations in the β subunit of B. subtilis could also confer StlR, we made three StlR substitutions (A499V, G500R, and E502V) in the rif region of rpoC. Together these results suggest that β and β’ interact to form an Stl binding site, and that this site is important for transcription elongation.

The RNA polymerase core enzyme of eubacteria has a multisubunit structure containing one β, one β’, and two α subunits (see Ref. 1 for a review). Most of the catalytic functions of the enzyme are thought to reside in the two largest subunits, β and β’. Because the β and β’ subunits share colinear blocks of conserved sequence with the two largest subunits of eukaryotic RNA polymerases, genetic and biochemical analysis of the eubacterial enzyme can contribute to an understanding of the structure-function relationships among RNA polymerases of all organisms (1).

One way to explore these structure-function relationships in eubacteria is to study the action of two antibiotics that specifically target RNA polymerase, rifampicin (RifR) and streptolydigin (Stl), each of which has a different mechanism of inhibition. Rif arrests transcriptional initiation at the promoter by locking RNA polymerase in an abortive initiation complex capable of synthesizing only short oligonucleotides, but this antibiotic has no effect once the transcription complex has elongated past the promoter (2). In contrast, Stl blocks both transcriptional initiation and elongation by inhibiting the translocation step, thereby reducing the rate of chain formation. This translocation inhibition has been suggested to result from interference with either the nucleotide triphosphate binding site or the ability to form the phosphodiester bond between the incoming triphosphate and the nascent RNA chain (3–5).

In the Gram-negative bacterium Escherichia coli, mutations that confer either Rif resistance (RifR) or Stl resistance (StlR) have been mapped to rpoB, the gene encoding the β subunit (6–10). Likewise, reconstitution studies in the Gram-positive bacterium Bacillus subtilis found that RifR is also associated with the β subunit, but that StlR is associated with β’ (11, 12). Because the primary sequences of β and β’ are highly conserved between E. coli and B. subtilis (13), it was reasonable to presume that additional sites for StlR might lie within the β’ subunit of E. coli and within the β subunit of B. subtilis.

Here we use in vitro mutagenesis to establish that additional sites for StlR in B. subtilis do map within rpoB, the gene encoding the β subunit of RNA polymerase. We also use molecular techniques to precisely map two previously isolated StlR mutations within rpoC, the gene encoding the β’ subunit of RNA polymerase. Both StlR mutations in rpoC alter the same residue in a region highly conserved among the largest subunits of prokaryotic and eukaryotic RNA polymerases. In the accompanying paper, Sev- erinov et al. (14) show that newly isolated alterations to StlR also affect the same conserved region in E. coli rpoC. Together with previous results, these data suggest that the β and β’ subunits of eubacterial RNA polymerase interact to form an Stl binding site.

EXPERIMENTAL PROCEDURES

Strains, Phage, and Genetic Methods—E. coli DH5a was host for plasmid constructions, and E. coli Y1090 was host for agt11 phage, grown as described previously (15). Streptolydigin, a gift of Laura Heisler and Carol Gross, was incorporated into tryptase blood agar base plates (Difco) at 3–50 μg/ml. Standard genetic and recombinant DNA methods were performed as described previously (13). Polymerase chain reactions (PCR) were done using Taq polymerase (Promega, Madison, WI) according to published protocols (16), and the resulting products were cloned into the pCR II vector (Invitrogen, San Diego, CA). DNA sequencing was done on subcloned fragments by one of two methods: (i) automated DNA sequencing using the Applied Biosystems 373A with analysis software version 1.2, or (ii) dideoxynucleotide chain termination with reactions primed on double-stranded DNA templates, using the Sequenase enzyme and protocols from U. S. Biochemical Corp. For direct genomic sequencing the fmol™ DNA Sequencing System (Promega) was used as specified by the manufacturer.

Isolation of the B. subtilis rpoC Gene—We previously isolated a agt11 phage that carried the 5’ third of rpoC (13). To obtain the remaining portion of rpoC, we first amplified a 2.2-kb PCR product from the B. subtilis genome using Primer A (5’-GAAGTTGGGATGACTAG-3’) from the available rpoC sequence together with degenerate Primer B (5’-
A different organization of the B. subtilis rpoC region—We previously described the isolation of the B. subtilis rpoB gene and the 5’ third of rpoC (13). To address the question of whether StlR could be conferred by alterations in either the β or β’ subunits of RNA polymerase, we first used chromosomal walking methods to isolate the remainder of rpoC and the downstream rpsL gene. As shown in Fig. 1, the resulting fragments carried overlapping regions of the B. subtilis chromosome, which bore the 3’ two-thirds of the β’ coding region.

Coding regions were identified by aligning the predicted B. subtilis products with their E. coli counterparts (25). As shown in Fig. 1, B. subtilis had a gene order similar but not identical to E. coli, with the rpoC homologue (encoding β’) followed by an open reading frame (orf82) that could encode a protein of 82 residues (P9). This P9 reading frame was in turn closely followed by the rpsL homologue (encoding ribosomal protein S12). The predicted sequence of P9 was significantly similar (28% identity in a 71-residue overlap; z value of 10.6 standard deviations above the mean of a shuffled sequence) to a hypothetical ribosomal protein encoded by orf104, which occupies a position downstream of the rpoC homologue in Sulfobolus aquaticus (26). However, the P9 reading frame is entirely absent from the equivalent E. coli region, where rpoC is directly followed by rpsL.

The proposed rpoC reading frame encodes a predicted 1,199-residue product that is 53% identical to E. coli β’ (17) and 55% identical to Mycobacterium leprae β’ (27). Moreover, all three β’ sequences share the eight regions (A through H) that are highly conserved among the largest subunits of eubacterial and eukaryotic RNA polymerases (nomenclature according to Ref. 28). Notably, as shown in Fig. 1, the β’ subunit from the Gram-positive B. subtilis lacks a 189-residue segment found between conserved regions G and H in the equivalent subunit of the Gram-negative E. coli. The β’ subunit from the Gram-positive M. leprae also lacks this segment (27). The complete absence of this segment from the Gram-positive lineage suggests that it is not essential for minimal β’ function.

StlR Mutations Map within a Highly Conserved Region of rpoC That Is Important for Transcriptional Elongation—Two spontaneous StlR mutations, stl445 and stl6, were previously isolated by A. L. Sonenshein and colleagues (22, 23), and they kindly supplied strains bearing these alleles. To first establish whether alterations in the β’ subunit could indeed confer StlR on B. subtilis RNA polymerase in vivo as suggested by subunit reconstitution experiments (12), we made three overlapping PCR fragments that carried the entire rpoC coding sequence. For these experiments, chromosomal DNA from the StlR strain MO34 (stl445) was used as a template. As shown in Fig. 1,
these overlapping fragments were carried by plasmids pXY11, pXY12, and pXY13. To identify the fragment containing the stl445 alteration, these plasmids were linearized so that they could replace the homologous rpoC region on the B. subtilis chromosome via a double crossover event. Upon transformation into the wild-type strain PB2, only plasmid pXY12 conferred StlR at a frequency significantly higher (50-fold) than the control plasmids, which bore the same three fragments isolated from the wild-type. StlR strain (not shown). We inferred from these results that the stl445 alteration lay within the 1-kb rpoC fragment that was unique to pXY12 and was not present on the other two plasmids (see Fig. 1).

To locate the stl445 alteration more precisely, we made three additional plasmids carrying portions of this 1-kb region. As shown in Fig. 1, these fragments were carried by plasmids pXY16, pXY19, and pXY21. After transformation into wild-type strain PB2, linearized plasmids pXY19 and pXY21 both yielded StlR transformants at a frequency significantly higher than the control plasmids. We concluded that the stl445 alteration lay within the 565-nt rpoC fragment common to both pXY19 and pXY21. Analysis of this fragment revealed only a single transition from the wild-type sequence, an A to a G at nt 7118. This transition would lead to substitution of a glycine for an aspartate at residue 796 of B. subtilis β′ (D796G). We directly sequenced the corresponding region of the MO34 (stl445) parent chromosome and confirmed that it was identical to wild type except for the A to G transition at nt 7118. Furthermore, this sequenced region again bestowed StlR when amplified from the MO34 genome and transformed into a StlR recipient. Similar transformation and sequencing experiments were performed using PCR fragments generated from strain MO38 (stl6). These experiments found that the stl6 allele caused the identical D796G substitution. Thus two independently isolated stl alleles targeted the same β′ residue. We conclude that the D796G substitution alone is sufficient to confer StlR in vivo. Notably, B. subtilis residue D796 lies in the C-terminal portion of Region F, one of the eight regions that are conserved among the largest subunits of eukaryotic RNA polymerases and E. coli β′ (28).

Fig. 2 shows a comparison of Region F in B. subtilis β′, E. coli β′, and representative eukaryotic homologues. Significantly, the available evidence indicates that Region F is important for transcriptional elongation in both prokaryotic and eukaryotic cells. Among eukaryotes, all known alterations that enhance resistance to the fungal toxin α-amanitin are found in or immediately adjacent to Region F (see Ref. 29 and references therein). α-Amanitin binds directly to RNA polymerase II and inhibits both transcriptional initiation and elongation, slowing phosophodiester bond formation and translocation by an as yet unknown mechanism (30–33). Among prokaryotes, substitutions at 14 of the 85 residues in E. coli Region F are known to alter the termination properties of the enzyme, and this consequence is thought to reflect the effects of these substitutions on elongation kinetics (34). Weilbaecher et al. (34) advanced a possible explanation for these various phenotypes; Region F might comprise part of a site that binds either the 3′ end of the nascent transcript or the new DNA template as it directs incorporation of incoming nucleotides, and disruption of either of these activities would inhibit the elongation reaction.

The results we report here and those in the accompanying paper (14) are consistent with this possibility. In addition to the StlR alteration we mapped in Region F of B. subtilis β′, Severinov and colleagues identified three alterations to Region F of E. coli β′ that confer StlR in vitro (14). One of these, S793F, targets the residue that corresponds to S797 in B. subtilis and lies immediately adjacent to the B. subtilis D796G alteration we mapped (see Fig. 2). The other two, M747I and R780H, are from the collection of termination-altering mutants selected by Weilbaecher et al. (34) and confer only weak StlR. StlR in prokaryotes might be considered the functional analog of α-amanitin in eukaryotes, in that both molecules affect transcription initiation and elongation primarily by interfering with the elongation reaction. If this is the case, then the similar location of alterations conferring StlR in organisms as evolutionarily diverse as B. subtilis and E. coli provides further evidence for the importance of Region F in the elongation reaction of RNA polymerases from all organisms.

StlR Mutations Also Map in the Conserved rif Region of rpoB—In E. coli β′, a number of substitution, deletions, and insertions in the region between Rif clusters I and II were found to confer StlR (6, 9). Further extensive mutagenesis found that single substitutions conferring StlR were confined to the four contiguous residues 543–546 (10), which correspond to residues 499–502 of B. subtilis β′ (13). Because the sequences of the two Rif clusters and the intervening spacer regions were so similar in the two species (Fig. 3), it was reasonable to expect that alterations in residues 499–502 of B. subtilis β′ would also lead to StlR.

Plasmids bearing five different alterations in this region were constructed as described under “Experimental Procedures” and introduced into the rpoB region of the B. subtilis chromosome by transformation. Only plasmids bearing the A499V, G500R, or E502V alterations yielded StlR transformants at a frequency significantly greater (10-fold) than the control plasmid, which bore the corresponding wild-type region. In contrast, plasmids bearing the M501S and Δ(499–502) alterations failed to yield significant numbers of StlR transformants. Direct sequencing of the chromosome of representative StlR strains verified that each of the alterations, A499V, G500R, and E502V, was the only change that had been introduced into the rpoB region. To determine whether the A499V, G500R, and E502V alterations were sufficient to confer StlR in vivo, the region containing each was amplified from the appropriate StlR strain by PCR, sequenced to confirm the fidelity of the amplification, then transformed a second time into a StlR.
consistent with a single site of interaction between the antibiotic and the subcortical core enzyme (5). Second, in both prokaryotic and eukaryotic systems, there is ample evidence which suggests that regions homologous to the putative Stl target in B. subtilis β’ are important in transcription elongation (29–34). And third, the putative Stl target region in E. coli β, although itself dispensable (9, 10), nonetheless lies immediately adjacent to regions implicated in nucleotide binding and transcription elongation (8, 38).

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