The Staphylococcus aureus ileS Gene, Encoding Isoleucyl-tRNA Synthetase, Is a Member of the T-Box Family

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The Staphylococcus aureus ileS gene, encoding isoleucyl-tRNA synthetase (IleRS), contains a long mRNA leader region. This region exhibits many of the features of the gram-positive synthetase gene family, including the T box and leader region terminator and antiterminator. The terminator was shown to be functional in vivo, and readthrough increased during growth in the presence of mupirocin, an inhibitor of IleRS activity. The S. aureus ileS leader structure includes several critical differences from the other members of the T-box family, suggesting that regulation of this gene in S. aureus may exhibit unique features.

The antibiotic mupirocin (pseudomonic acid) is a potent inhibitor of isoleucyl-tRNA synthetase (IleRS), which is responsible for charging of isoleucyl-tRNA with isoleucine (17). Bacterial resistance to mupirocin can be caused by IleRS enzymes with altered affinity for the drug (4, 6, 28). The chromosomal ileS gene, encoding IleRS, was recently isolated from Staphylococcus aureus, one of the pathogenic organisms against which mupirocin is used clinically (3). Examination of the nucleotide sequence of the region immediately upstream of the IleRS coding sequence revealed the presence of a perfect T-box sequence, an element which is conserved in a number of aminoacyl-tRNA synthetase and amino acid biosynthesis genes in Bacillus subtilis and other gram-positive organisms (12, 14). The genes in this group are regulated by a common transcription antitermination mechanism, in which the cognate uncharged tRNA acts as an effector to stimulate readthrough of a termination signal located in the mRNA leader region of each gene or operon (10). The specificity of the mRNA-tRNA interaction is mediated at least in part by two base-paired regions: a triplet sequence in the mRNA, designated the specifier sequence, which pairs with the anticodon of the cognate tRNA; and a bulged portion of an antiterminator structure in the tRNA (5′-UCCN-3′), which interacts with the 3′ end of the tRNA (5′-NCCA-3′). The antiterminator and terminator are mutually exclusive structures, and interaction between uncharged tRNA and the antiterminator is proposed to promote or stabilize antiterminator formation, preventing termination (10, 13).

The ileS gene is the first chromosomal aminoacyl-tRNA synthetase gene sequence to be identified in S. aureus (3). In this study, additional sequence upstream of the S. aureus ileS coding region was determined, and ileS transcription was characterized. The region upstream of the IleRS coding sequence was examined for similarity to the complex pattern of primary sequence and structural features common to the other T-box genes. While there are some interesting deviations from the Bacillus-type pattern, the S. aureus ileS gene contains most of these conserved features, suggesting that it is likely to be regulated through this transcription antitermination mechanism.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Strains were routinely propagated at 37°C in LB medium (21) or tryptose blood agar base (Difco) for B. subtilis. For Escherichia coli plasmid-containing strains, ampicillin was used at 50 μg/ml, and for B. subtilis, chloramphenicol was used at 5 μg/ml for selection for the SPI prophage, which contains a cat gene, encoding chloramphenicol acetyltransferase. E. coli DH5α was used for plasmid propagation; strain JM103 was used for propagation of phage M13.

Isolation and characterization of the ileS upstream region. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs or Promega and were used according to the manufacturer’s instructions. DNA sequencing was carried out by dideoxynucleotide sequencing using Sequenase 2.0 (United States Biochemical). PCR was carried out by using AmpliTaq DNA polymerase (Perkin-Elmer/Cetus). Oligonucleotides were purchased from DNA International. Chromosomal DNA was isolated from S. aureus Oxford by a modification of a previously described protocol (15), with the substitution of lysozyme (50 μg/ml; Sigma Chemical Co.) for lysozyme.

The original ileS clone, pBROC461, contained an 8.5-kb fragment of S. aureus DNA, including 0.6 kb upstream of the sequence previously reported (3). The sequence of the remaining portion of the clone, which extended to the TtpA site at position 255 in Fig. 1, was determined, and an overlapping upstream fragment was isolated by using inverse PCR. S. aureus chromosomal DNA (0.1 μg) was digested to completion with Sau3A1 and ligated in low concentration to form intramolecular circles, which were PCR amplified with oligonucleotides STAD3 and STAD4 (Fig. 1) as primers. The resulting DNA product was digested with Sau3A1 and PstI, and the 0.9-kb fragment was inserted into the bacterial plasmid M13 mp18 for DNA sequencing. Four independent isolates were sequenced to ensure that no alterations had arisen during amplification.

Transcriptional analysis. For primer extension analysis, total RNA was isolated as described by Sandler and Weisblum (26) from S. aureus cultures grown to late logarithmic growth phase in LB broth. Oligonucleotides STAD1, STAD2, and STAD3 (Fig. 1) were 5′ end labeled with [32P]ATP, hybridized to 20 μg of RNA, and extended by using avian myeloblastosis virus reverse transcriptase. Extension products were resolved on a denaturing 6% polyacrylamide gel, using DNA sequencing reactions primed with the same oligonucleotides as size standards, and visualized by autoradiography.

For Northern analysis, S. aureus was grown to an A600 of 0.3, and mupirocin was added to half of the culture to a final concentration of 10 ng/ml. Samples (25 ml) were taken from each culture at 30, 60, 90, and 120 min following the addition of mupirocin and added directly to ice-cold TE (50 mM Tris-HCl [pH 8], 1 mM EDTA). Cells were collected by centrifugation, and total RNA was isolated. RNA samples (10 μg) were electrophoresed, blotted, and hybridized by using NorthernMax reagents (Ambion) as instructed by the manufacturer. An antisense biotinylated riboprobe was generated with BrightStar BIOTINscript (T7 kit; Ambion), using plasmid pGEM7SauIleS, which contains the 580-bp NsiI-EcoRI fragment of ileS DNA from pBROC461 (3), linearized at the MluI site (position 742 in Fig. 1). The riboprobe (360 nucleotides [nt]) included 200 nt of 5′ noncoding sequence and extended 360 nt into the coding region. Chemiluminescent signals were developed by using BrightStar BioDetect (Ambion) as instructed by the manufacturer.

Transcriptional fusions to lacZ were constructed by using plasmid pFG328, which contains a promoterless lacZ gene (11). All fusion constructs contained the B. subtilis rpd3 promoter but lacked the target site for autogenous regulation by...
ribosomal protein S4 (9). The pS4LES construct contained the rpsD promoter as a 200-bp HindIII-PstI fragment derived from plasmid pTMH131, with the rpsD Pst-1 mutation at 171 relative to the rpsD transcription start point, and a 660-bp NsiI-EcoRI fragment of ileS DNA from pBROC461. The pS4ILESK construct was derived from pS4ILES, with the removal of the KpnI-EcoRI region (from the T box into the ileS coding region). Fusions were introduced into specialized transducing phage SPb as previously described by transformation of B. subtilis ZB307A, and fusion phage were purified by passage through strain ZB449 before introduction into strain 1A231. Cells were grown in LB medium to early exponential growth phase and then were split, and various concentrations of mupirocin were added. Cells were harvested after 2 to 3 h, and β-galactosidase activity was measured after toluene permeabilization as described by Miller (21).

**RESULTS**

**Sequence of the ileS upstream region.** The original ileS clone contained 0.6 kb of DNA upstream of the published sequence (3). An overlapping Sau3A1 DNA fragment was obtained by inverse PCR, and the sequence of a 0.85-kb region was determined (Fig. 1). Analysis of this region revealed the presence of an open reading frame (ORF) encoding 205 amino acids upstream of the IleRS coding region. A sequence with excellent adherence to the consensus for translational start sites in gram-positive bacteria (AAGGAGG) was identified at an appropriate position upstream of an AUG initiator codon at the start of the ORF. This ORF is closely related to the B. subtilis divIVA gene, a cell division gene which is also located immediately upstream of ileS (2). The ileS genes of E. coli (19) and Methanobacterium thermoautotrophicum (18) are also preceded by ORFs of unknown function, encoding 312 and 401 amino acids, respectively; these upstream ORFs exhibit no obvious similarity to each other, to B. subtilis divIVA, or to the ORF upstream of S. aureus ileS.

**Start point of ileS transcription.** The ileS transcription start point was located by primer extension (Fig. 2). We identified a

**TABLE 1. Bacterial strains used**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Prototroph</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus Oxford</td>
<td>Prototroph</td>
<td>S. aureus Oxford Prototroph American Type Culture Collection</td>
<td>29</td>
</tr>
<tr>
<td>B. subtilis</td>
<td></td>
<td>ZB307A SPk2del2::Trn917::pSK10Δ6</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZB449 trpC2 pheA1 abh7803 (SPk cured)</td>
<td>Bacillus Genetic Stock Center, Ohio State University</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1A231 ilvA1 trpC2</td>
<td>23</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td>DH5a p80lacZΔM15 endA1 recA1 hsdR17 (rK− mK−) thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JM103 endA1 supE44 sbeBC thi-1 rpsL Δ(lac-pro)/F' traD36 lacFΔZΔM15 proAB</td>
<td>20</td>
</tr>
</tbody>
</table>

**Nucleotide sequence accession number.** The sequence data shown in Fig. 1 have been deposited in the EMBL/GenBank/DDBJ nucleotide sequence data libraries under accession no. U41072.
single start point, corresponding to position 277 on the sequence shown in Fig. 1, 677 bp upstream of the first position of the AUG translational start codon for ileS. No other transcripts with 5' ends downstream of this position were identified by using primers (STA1, STA2, and STA3 [Fig. 1]) throughout the region upstream of the IleRS coding sequence (data not shown). Position 277 is preceded by a sequence with a −35 region of TTGAAA (five-of-six match to the *E. coli* Es70 and *B. subtilis* EsA consensus) and a −10 region of GATACT (four-of-six match to the consensus), with a 17-bp spacer. This site is located within the coding sequence for the upstream ORF so that transcripts initiating at this position would not encode the product of the upstream ORF. The possible existence of additional transcripts initiating further upstream is not ruled out.

**Structural model of the ileS mRNA leader region.** Many aminoaacyl-tRNA synthetase genes in gram-positive bacteria exhibit long leader mRNAs, with extensive conservation of primary sequence and secondary structure elements which have been shown to be important for regulation (14, 25). The most highly conserved sequence element in this family of genes is the T box, a 14-nt element with the consensus sequence 5'-AGGGUGGNACCGCG-3', which is usually located upstream of a factor-independent transcription termination site in the leaders (16); the N represents a variable position which pairs with the discriminator base at the 3' end of the tRNA (13). A perfect T-box sequence is present at positions 872 to 885 in the ileS sequence in Fig. 1, 45 to 58 bp upstream of the IleRS ribosome binding site.

This region was inspected for the presence of an inverted-repeat sequence resembling an intrinsic transcription termination signal. A large inverted repeat was found extending from positions 852 to 917, and this putative stem-loop is followed by a run of U residues, as is characteristic of intrinsic termination signals (Fig. 3A). In all of the genes in the T-box family, an antiterminator structure is formed by pairing of a portion of the T-box sequence with a conserved sequence on the left side of the terminators, so that the terminator and antiterminus are mutually exclusive. The ileS leader varies from this pattern in that the antiterminator-like structure is present within the terminator-like structure (Fig. 3B). Two alternate structures which vary in the presence or absence of extended
pairing below the antiterminator structure can be formed, so that only the terminator form contains this additional paired region. This finding suggests that the mechanism for control of antitermination may be different in this gene compared to the other members of the T-box family. In this case, interaction with the tRNA would be expected to prevent the pairing in the region below the antiterminator rather than promoting formation of an alternate structure.

In the canonical structures, the side bulges of the antiterminator structures have the highly conserved sequence 5'-UGG NACC-3', where N is a variable position, and the 5'-UGGN-3' pairs with the 5'-NCCA-3' at the acceptor end of the corresponding tRNA; the N positions in the mRNA and the tRNA covary, so that base pairing is maintained (13). In ileS, the antiterminator sequence is 5'-UGGU-3', which corresponds with the 5'-ACCA-3' present at the acceptor end of isoleucyl-tRNAs from both B. subtilis (7, 27) and S. aureus (8).

The leader regions of the other T-box genes all contain three major stem-loop structures upstream of the T-box sequence. Stem I is the largest and most complex structure and contains the specifier sequence triplet which pairs with the anticodon of the cognate tRNA (10). This sequence is a codon, in general the specifier sequence triplet which pairs with the anticodon of the cognate tRNA; the N positions in the mRNA and the tRNA covary, so that base pairing is maintained (13). In ileS, the antiterminator sequence is 5'-UGGU-3', which corresponds with the 5'-ACCA-3' present at the acceptor end of isoleucyl-tRNAs from both B. subtilis (7, 27) and S. aureus (8).

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Growth of S. aureus in the presence of mupirocin, an inhibitor of IeRS activity, is predicted to result in accumulation of uncharged tRNAleS. Northern analysis (Fig. 4) was used to determine if the leader region terminator of ileS is functional in S. aureus and to examine the effect of mupirocin on readthrough of the terminator. The amount of the full-length transcript (3.6 kb) increased over 10-fold in response to mupirocin. A slight decrease in the abundance of the terminated transcript (0.65 kb) was observed in response to the antibiotic. Intermediate-size hybridizing bands may represent degradation products of the readthrough ileS transcript, possible overlapping transcripts, or cross-hybridization of the probe to tRNA. Similar results were seen in assays using a shorter probe, lacking 130 nt of the 5' noncoding sequence (data not shown); this probe resulted in a weaker signal for the 0.65-kb band, supporting its identification as the terminated transcript.

Expression of ileS-lacZ fusions in B. subtilis was also tested. These constructs contained the strong B. subtilis rpsD ribosomal protein gene promoter, so that the effect of the leader sequences on termination and readthrough could be assessed without concerns about the efficiency of the heterologous S. aureus promoter. Plasmid pS4ILES containing the NsiI-EcoRI ileS region (starting just upstream of the putative stem II structure at position 663 and extending to position 1300, within the coding region) gave 11 to 13 U of β-galactosidase activity (data not shown). Deletion of the region downstream of the KpnI site at position 880 (pS4ILESK) was predicted to destroy the putative transcriptional terminator; this fusion yielded a sevenfold increase in lacZ expression. The effect of growth in the presence of a low amount of mupirocin on ileS-lacZ expression was tested, since mupirocin is expected to cause accumulation of uncharged isoleucyl-tRNA. Mupirocin concentrations from 5 to 200 ng/ml were tested, with various treatment times; the MIC of mupirocin for B. subtilis is 100 ng/ml (1). Maximal induction was observed with 20 ng/ml, at 2 to 3 h; no inhibition of growth was observed under these conditions. The fusions containing the terminator exhibited reproducible 1.8-fold induction of ileS-lacZ expression, while there was no effect on expression of the fusion lacking the terminator or on expres-
sion of a control tyrS-lacZ fusion (data not shown). An identical pattern was observed with an ileS-lacZ translational fusion (data not shown), suggesting that the effect is mediated at the level of transcription. The differences between the S. aureus ileS leader and the leaders of the B. subtilis genes in this family may be responsible for poor inducibility of the S. aureus ileS gene in B. subtilis.

**DISCUSSION**

The ileS gene of S. aureus is a member of the T-box family of genes in gram-positive bacteria, which are regulated at the level of transcription antitermination in response to amino acid limitation. This is the first member of this family from a pathogenic organism. The ileS mRNA leader region contains most of the conserved sequence and structural elements found in the members of this family, including the T box, terminator and antiterminator, and the critical isoleucine codon embedded in an appropriate position in stem I. The ileS leader differs from those of other members of the family in a number of ways, including the location of stem II upstream of stem I instead of downstream, the absence of the highly conserved F-box sequence normally found between stems II and III, and the position of the T box within the terminator rather than upstream. These structural differences may reflect interesting variations in the mechanisms of transcription antitermination of T-box genes in different gram-positive species, despite the apparent overall conservation of this regulatory mechanism.

The most unusual feature of the S. aureus ileS leader is the location of a canonical antiterminator-like structure at the top of the terminator structure. In all members of this group, the sequences which form the right side of the antiterminator also form the 5′ half of the terminator, so that the terminator and antiterminator structures are mutually exclusive (10, 14). In contrast, the terminator and antiterminator conformations of S. aureus ileS differ in the presence or absence of extended base pairing below the antiterminator structure; in the antiterminator form, the region which can form the 3′ half of the base of the terminator is instead captured in a separate stem-loop structure (Fig. 3B). The model for the other genes in this group is that interaction of the 3′ end of the cognate uncharged tRNA with the side bulge of the antiterminator causes or stabilizes antiterminator formation (10, 14). For ileS, this interaction is still postulated to occur, but it appears likely that an additional interaction, with tRNA or with some other factor, is necessary for formation of the stem-loop upstream of the antiterminator, so that this region is unavailable for terminator formation. If this model (Fig. 5) is correct, then the molecular mechanism for antitermination is likely to be somewhat different for ileS than for the other genes in this family. It will be interesting to determine if this pattern is a general feature of S. aureus T-box family genes or is specific to ileS.

The ileS leader is also unique among the T-box genes in that there is an ORF which overlaps with the 5′ portion of the proposed regulatory sequences (stem II). The B. subtilis gltX-cysE-cysS operon, which includes a T-box regulatory site between gltX and cysE, also exhibits a coding region upstream of the termination site, but in that case the GltX coding region ends before the start of the regulatory elements (5). It appears that the major transcripts encoding ileS initiate at a site within the upstream ORF and therefore do not encode this ORF; there may be other transcripts initiating further upstream which do encode this product. This ORF is conserved in B. subtilis, where it has been identified as the divIVA locus (2).

Since S. aureus ileS is a member of the T-box family, expression would be predicted to be induced in response to limitation for isoleucine or inhibition of IleRS enzyme activity, since accumulation of uncharged tRNA<sub>ile</sub> is expected to occur in both cases. A greater than 10-fold induction of the readthrough ileS transcript in response to mupirocin, an inhibitor of IleRS, was demonstrated by Northern analysis of S. aureus mRNA. Induction of S. aureus ileS-lacZ expression by mupirocin in B. subtilis was less than twofold. The failure to observe strong induction in B. subtilis could be due to the differences in structure between the S. aureus leader and the B. subtilis genes in this family, so that the B. subtilis machinery fails to function efficiently with the S. aureus gene. There may be specific regulatory factors which are necessary for antitermination, and the variations in leader region structure may reflect variability in the properties of these factors. Alternatively, there may be significant differences in the structure of tRNA<sub>ile</sub> in the two organisms. One S. aureus tRNA<sub>ile</sub> gene sequence has been reported so far (7); this tRNA has a “C”AU anticodon, where the “C” represents an unknown modified base that probably results in pairing with AUA, rather than the GAU anticodon for interaction with the ileS AUC isoleucine specifier sequence. In B. subtilis, a tRNA<sub>ile</sub> gene with a GAU anticodon has been reported (27), but it is possible that B. subtilis tRNA<sub>ile</sub> is unable to interact appropriately with the S. aureus ileS leader. B. subtilis ileS appears to be a member of the T-box family (2), with a normal Bacillus-type leader structure and AUC isoleucine specifier sequence.

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**REFERENCES**

