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Regulatory Region of the *Klebsiella aerogenes* Tryptophan Operon

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The *trp* operon of *Klebsiella aerogenes* was cloned, and its regulatory region was sequenced. Comparison with previously reported *trp* regulatory sequences of other enteric bacteria indicates that the *K. aerogenes trp* promoter-operator region is most similar to the corresponding region of *Salmonella typhimurium*. The *trp* leader regions of *K. aerogenes* and other enteric bacteria are organized similarly, but there are significant differences in the stabilities of the predicted secondary structures in their leader transcripts. These differences should make the *K. aerogenes* attenuator a weaker transcription termination site than any of the other attenuator regions studied; this was confirmed in in vitro transcription experiments. The sequence of the leader transcript and the precise site of in vitro termination were determined.

In enteric bacteria, the expression of the *trp* operon is regulated by two distinct mechanisms, repression and attenuation (22). Comparison of the nucleotide sequences of the regulatory regions of the *trp* operon of several enteric bacteria and studies with regulatory mutants of these organisms have revealed how repression and attenuation are used to regulate *trp* operon expression. In the presence of tryptophan the *trp* repressor binds at an operator site within the *trp* operon promoter (5, 16). Bound *trp* repressor prevents binding of RNA polymerase, thereby inhibiting transcription initiation (18). The leader segment, located between the transcription start site and the beginning of the first structural gene, contains an attenuator, a site at which transcription termination is regulated. The transcript of the leader region can form either of two secondary structures, one of which causes transcription termination at the attenuator. Which of the two structures the transcript assumes is thought to depend on the position of the ribosome translating the leader transcript, and this is in turn regulated by the availability of charged $tRNA^{Trp}$ in the cell (22). In this paper we present a comparison of the regulatory region of the *trp* operon of *Klebsiella aerogenes* with those of other enteric bacteria and discuss the mechanistic features relevant to attenuation control that are peculiar to the *K. aerogenes trp* operon.

MATERIALS AND METHODS

Procedures used for cloning *K. aerogenes trp* DNA and mapping restriction sites have been described previously (10). DNA sequencing was performed by the Maxam and Gilbert method (8). In vitro transcription reactions were carried out as described previously (21), except that 7 M urea–8% acrylamide gels were used in the analysis of transcription products. The relative extent of transcription read-through by RNA polymerase was measured in experiments in which a single round of transcription was allowed for each RNA polymerase-promoter complex (21); reaction mixtures lacking UTP and CTP were pre-equilibrated at 37°C, and the reactions were initiated by the addition of a solution containing these triphosphates and rifampicin. RNase T_1 digestion conditions, fingerprint analysis of oligonucleotides, digestion with pancreatic RNase, and electrophoresis on DE 81 paper were by standard procedures that have been described previously (1, 3).

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RESULTS

Isolation, mapping and sequencing of plasmids containing *K. aerogenes trp* genes. Fragments generated by restriction of *K. aerogenes* DNA with *Hin*dIII or *Bam*HI endonucleases were ligated into the corresponding sites of vector pBR322. The mixtures were used to transform a *trpE Escherichia coli* strain, CY15002 (23), to prototrophy. Two plasmids, pKA1 and pKA2, were isolated; they contained inserts of 13.5 and 11.2 kilobases respectively (Fig. 1). Plasmid pKA2 was found to have an incompletely digested *K. aerogenes* DNA fragment. Subcloning *Bam*HI fragments of pKA1 into pBR322 yielded pKA3. Whereas plasmids pKA1 and pKA2 contain the entire *trp* operon, pKA3 lacks a portion of *trpA* (13). This observation established the presence of a *Bam*HI site within *trpA*.

The single *Hpa*I site in *K. aerogenes trp* DNA

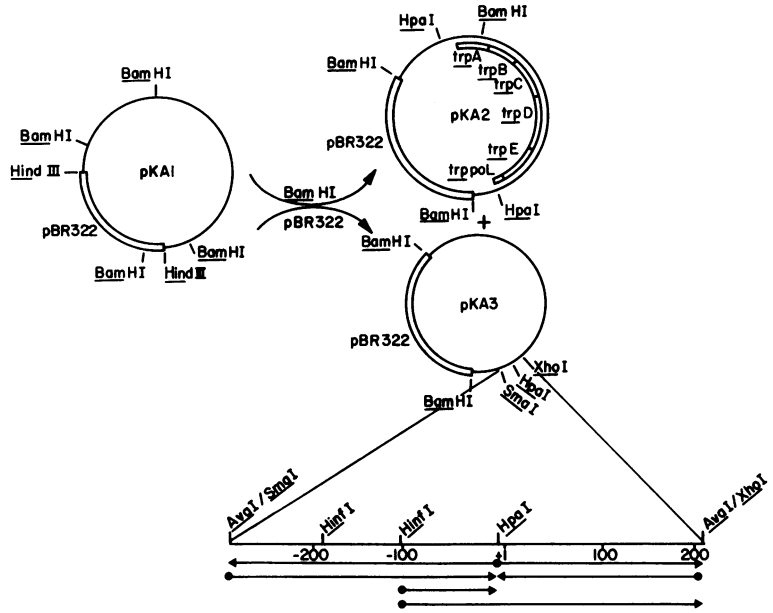

FIG. 1. Construction and maps of *K. aerogenes trp* plasmids. The double lines represent pBR322 vector DNA. The arrows at the bottom indicate the direction and extent of sequencing runs that were used to determine the entire sequence of the 481-bp *Ava*I fragment. The transcription start was numbered +1.

is about 6.5 kilobases upstream from *trpA*. This site is protected from restriction by *Hpa*I endonuclease by preincubation with RNA polymerase (data not shown). Since most enteric *trp* promoters contain an *Hpa*I site and since the single *Hpa*I site in the cloned *K. aerogenes* DNA is protected by polymerase, it is likely that the *Hpa*I site is within the *trp* promoter. In *K. aerogenes* this *Hpa*I site is located on a convenient 480-base-pair (bp) *Ava*I fragment. This fragment was isolated, and both strands were sequenced (Fig. 1).

```
K.AEROGENES
                -40        -30
        TCTGCAAACAAGGGTTGACTTTATTCCATTGAACTAGTTAACTAGTACGC
        A       T G T      A         C                   A
S.TYPHIMURIUM

         10         20      MET ALA MET HIS PHE ILE THR LEU HIS SER TRP TRP ARG THR SER END        80
AAGTTCACATGAAG GGGTATCACG ATG AAA ATG CAC TTT ATC ACT CTG CAC AGC TGG TGG CGC ACC TCC TGA CGACGGGCG
              A         T  AA  TG GCA GCG ACA T T G A T A     G T               T         TAG
                           MET ALA ALA THR PHE ALA PHE     GLY

        90        100        110        120        130        140
GCGTGATCGCGTTTTGCATTCAGCATACAGATACCCGGCCCGCC AATGAGCGGGCTTTTTATT
 T  ATGAA AGC GTA  CAGC  A                  TGT A             T

                                TRPE
   150        160        170        MET GLN THR SER LYS PRO
GCACAAAAATAATACGAACAGGCGAGAACAATA ATG CAA ACA TCC AAA CCG
A       [redacted]          U  CC              C A       C
                                               PRO
```

FIG. 2. Comparison of the sequence of the *trp* regulatory regions of *K. aerogenes* and *S. typhimurium*. Only differences in the *S. typhimurium* sequence are shown; bars identify base pairs not found in *S. typhimurium*. Horizontal arrows denote dyad symmetry in the operator region. Vertical arrows mark the in vitro transcript termini.

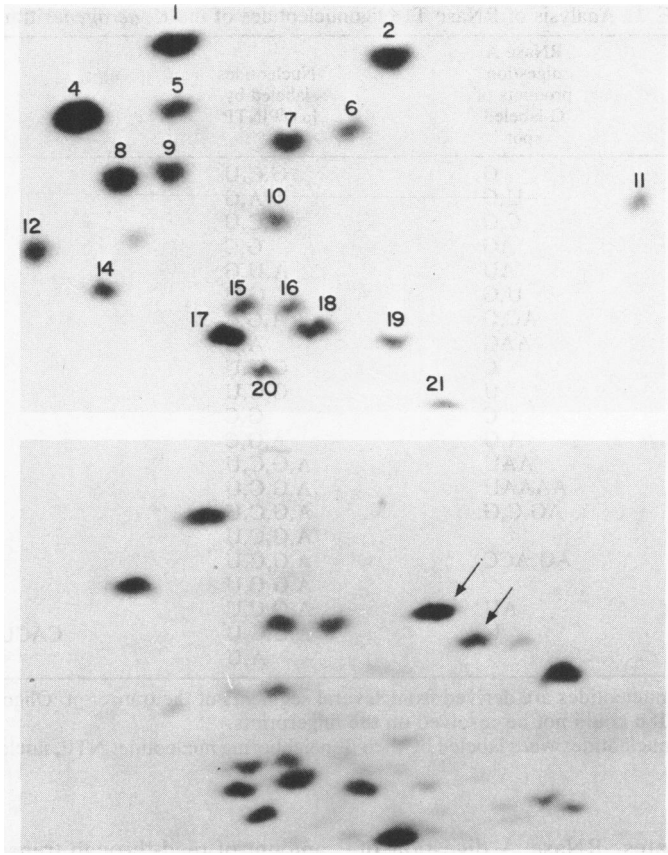

FIG. 3. Fingerprint analysis of the leader transcript labeled with [α-^{32}P]GTP (A) and [α-^{32}P]UTP (B). The arrows indicate the two 3′ oligonucleotides. Spot numbers correspond to the numbers in Table 1.

A portion of the sequence of the *Ava*I fragment containing the *K. aerogenes trp* regulatory region is presented in Fig. 2. The sequenced region extends 270 bp upstream from the presumed start of transcription and 200 bp into the transcribed region. On the basis of the sequence, we assume that this fragment contains the *trp* promoter, operator, leader region, and 20 bp of the first gene, *trpE*. As shown in Fig. 5, this region of *K. aerogenes* DNA has extensive sequence homology with the same region of other enteric bacteria. Particularly striking is the similarity of the *K. aerogenes* promoter-operator with the equivalent region in *S. typhimurium* (2); only seven differences exist in the 50 bp upstream from the presumed transcription start site. This region contains a segment with dyad symmetry centered between −12 and −11 which is undoubtedly the operator (5).

In vitro transcription of the *K. aerogenes* leader region. A transcript 144 nucleotides in length is produced during in vitro transcription of the *Ava*I fragment. The sequence of this transcript corresponds exactly to its DNA template. RNase T1 digests of the transcript gave a number of oligonucleotide spots (Fig. 3) that were further partially characterized by digestion with RNase A and by differential labeling with each of the four ribonucleoside triphosphates (Table 1). The 5′-terminal nucleotide of the transcript was shown to be A by incorporation of radioactivity from a γ-labeled nucleoside triphosphate. Although either one or both of the two adjacent A residues could be the 5′-terminal nucleotide, we designated the transcription start at the same position as in *E. coli* (19), since the *K. aerogenes* and *E. coli* sequences are identical between positions −20 and +8.

Two 3′-terminal spots were found in fingerprints of RNase T1 digests. These were the only spots that were not labeled by [^{32}P]GTP. However, they were labeled by [^{32}P]UTP (Fig. 3B). When the concentration of UTP used in the transcription region is high and the same as that of the other three nucleotides, the yield of the two spots is roughly equimolar; if the UTP concentration is lower, however, the spot corresponding to the shorter of the terminal oligonu-

TABLE 1. Analysis of RNase T1 oligonucleotides of the *K. aerogenes* transcript

Oligonucleotide[a]	RNase A digestion products of G-labeled spot	Nucleotides labeled by [α-^{32}P]NTP[b]	Sequence
1	G	G,C,U	G
2	U,G	A,G	UG
4	C,G	G,C,U	CG
5	AG	G,C	AG
6	AU	A,U,G	AUG
7	U,G	G,U	CUG
8	AC,G	A,G,C	ACG
9	AAG	A,G	AAG
10	C	G,C,U	AUCG
11	U	G,C,U	UUUUG
12	C	G,C	CCCG
14	A,G	A,G,C	CACAG
15	AAU	A,G,C,U	CCAAUG
16	AAAAU	A,G,C,U	AAAAUG
17a	AG,C,G	A,G,C,U	CAUACAG
17b		A,G,C,U	AUACCCG
18a	AG,ACC	A,G,C,U	CAUUCAG
18b		A,G,C,U	UAUCACG
19	AU	A,G,C,U	UUCACAUG
20	U	A,G,C,U	CACUUUACUACUCUG
3′		A,U	CUUUUUAU

[a] Some of the oligonucleotides are derived from several segments of the transcript. Oligonucleotide pairs 17a and 17b and 18a and 18b could not be resolved on the fingerprints.

[b] Some of the oligonucleotides were labeled by their 3′ neighboring nucleotide. NTP, nucleoside triphosphate.

cleotides predominates. RNase A digestion of the larger of the two fragments gave Cp, Up, and ApUp; therefore, its sequence must be CpUpU-pUpUpUpApUpU. Digestion of the smaller 3′-terminal fragment with RNase A gave Cp and Up and a new labeled oligonucleotide. This oligonucleotide, presumably ApU, did not correspond to any of the common oligonucleotides on DE 81 paper; it traveled to a position between Cp and Gp. From these data we conclude that RNA polymerase terminates transcription at the two positions marked by arrows in Fig. 2.

Transcription termination in vitro with *K. aerogenes* template DNA is not efficient. With *E. coli* and *Salmonella typhimurium* templates about 3 to 5% read-through was observed, whereas with *K. aerogenes* templates read-through ranged from 55 to 62% (Fig. 4). Read-through was calculated by excising labeled gel bands, counting them, and correcting for the number of G residues (21). The identity of the *K. aerogenes* read-through transcript was confirmed by RNase T_1 fingerprint analysis (data not shown). The addition of the rho termination protein to the reaction mixture had no effect on read-through, nor did transcription with RNA polymerase from mutant strain *rif-15* (12) (data not shown). Changes in the nucleoside triphosphate concentration had no effect on the relative amount of read-through transcript. At low temperatures, RNA polymerase pausing (21) around bp 90 was quite evident (Fig. 4).

DISCUSSION

The *trp* promoter-operator region of *K. aerogenes*. The *K. aerogenes trp* promoter is recognized by *E. coli* RNA polymerase as shown both by restriction site protection studies and by in vitro transcription analyses. The sequence of the promoter is virtually identical to that of *S. typhimurium* (Fig. 2). Only 7 of the 50 bp preceding the transcription start site are different. The dyad symmetry present in other promoters regulated by the *trp* repressor is also found in the *trp* promoter of *K. aerogenes*. This observation is in agreement with the previous finding that the *K. aerogenes trp* operon can be regulated in vivo by the *trp* repressor from *E. coli* and vice versa (7). Comparison of the 200 bp upstream from the promoter with the corresponding sequence of *E. coli* shows extensive homology (68%), albeit with many deletions or insertions (data not shown).

***K. aerogenes* leader region.** The leader sequence of the *trp* operon of *K. aerogenes* is very similar to that of other enteric bacteria (Fig. 5). DNA fragments containing the leader region are transcribed in vitro by *E. coli* RNA polymerase

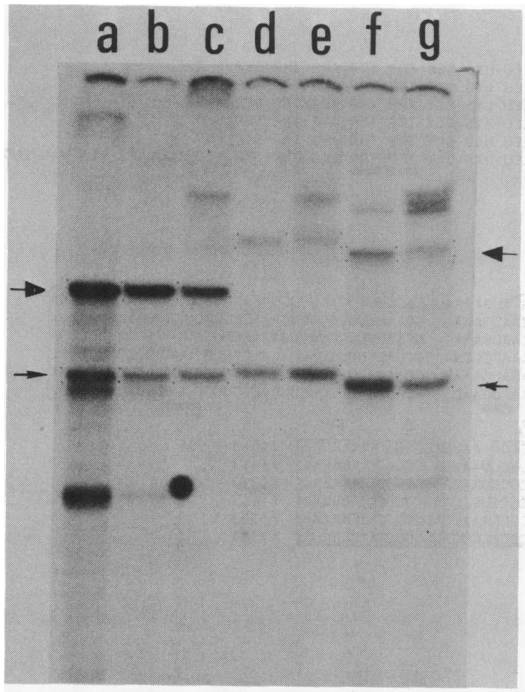

FIG. 4. In vitro transcription of *trp* leader templates: a, b, and c, *K. aerogenes*; d and e, *E. coli*; f and g, *S. typhimurium*. Only one round of transcription was allowed for each RNA polymerase-DNA complex. Lanes b, d, and f contain transcripts synthesized at 30°C; lanes c, e, and g contain transcripts synthesized at 37°C; and lane a contains transcript synthesized at 16°C with an increased amount of RNA polymerase. The large arrowheads mark the readthrough bands, and the small arrows mark the terminated leader transcripts. The rapidly moving band in lane a is the pause species.

to give a 144-nucleotide transcript. The sequence deduced from analyses of this transcript is in perfect agreement with the sequence predicted from the DNA sequence. The 5′ nucleotide of the transcript is an adenylic acid residue. We designate the first of a pair of adenylates as number one by analogy with the *E. coli* sequence (19). The 3′-terminal run of Us in the transcript is unlike the 3′-terminal sequence in *E. coli* and *S. typhimurium*: the run of Us is broken by a single A (Fig. 2). Transcription in vitro is terminated beyond this A after either of the next two Us. When low concentrations of UTP are used in the in vitro reaction, the first U is favored as a 3′ terminus.

The leader transcript encodes a 13- or 15-residue peptide, depending on which of two in-phase methionine residues is amino terminal. Comparison of the nucleotide sequence preceding the first methionine codon with the analogous sequence encoding the leader peptides of *E. coli* and other enteric bacteria suggests that translation begins at the first methionine codon (Fig. 5). The *K. aerogenes* leader polypeptide sequence contains adjacent tryptophan residues at the same positions relative to the hypothetical leader transcript secondary structures as in transcripts from the other enteric bacteria.

Leader transcript secondary structures. Comparison of the sequences of the leader transcripts of various enteric bacteria reveals highly conserved segments (Fig. 5). One conserved region is at the 3′ terminus of the transcript, where, in a segment of 15 nucleotides, the six organisms differ at only three positions. The 5′ portion of this segment can form a stable secondary structure with the G+C-rich block of high homology that is five to six nucleotides upstream. Such secondary structures followed by a run of Us often are at the 3′ end of procaryotic transcripts (15). All of the numbered highly conserved blocks indicated in Fig. 5 can participate in stable secondary structures. The two alternative secondary structures that can be predicted for the *K. aerogenes* transcript are shown in Fig. 6. The structure in Fig. 6A contains the 3:4 stem, the presumed terminator signal (22). This structure also contains the 1:2 stem, which is the first base-paired structure that can form in the nascent RNA molecule (14). The 5′ arm of this stem (segment 1 in Fig. 5 and 6) extends from the tryptophan codons to the leader polypeptide stop codon. Ribosomes translating this segment of the transcript could stall at the UGG codons when the cell is deficient in Trp-tRNA^{Trp} (6). This would prevent segment 1, sequestered by the ribosome, from base pairing with segment 2, allowing the 2:3 stem, the antiterminator, to form, as shown in the alternate structure in Fig. 6B. Segment 3 would thus be unavailable for the formation of the terminator structure. In this manner a deficiency of charged tRNA^{Trp} could prevent formation of the terminator and thereby increase operon expression.

Two additional potential base-paired structures, 5:6 and 7:8, are shown in Fig. 6. These can form regardless which of the alternative structures the RNA assumes. Segments 7 and 8 are not among the highly conserved sequences shown in Fig. 5; of the other five sequenced *trp* leader transcripts, only that of *Serratia marcescens* can form a similar base-paired structure (11). The function of 7:8 is unclear. Segment 5 (Fig. 5 and 6) contains the ribosome binding site that is presumably used in the synthesis of the leader peptide. In every enteric *trp* leader sequence known, this region can form a stable base-paired structure with segment 6. The role of this base pairing could be to prevent multiple

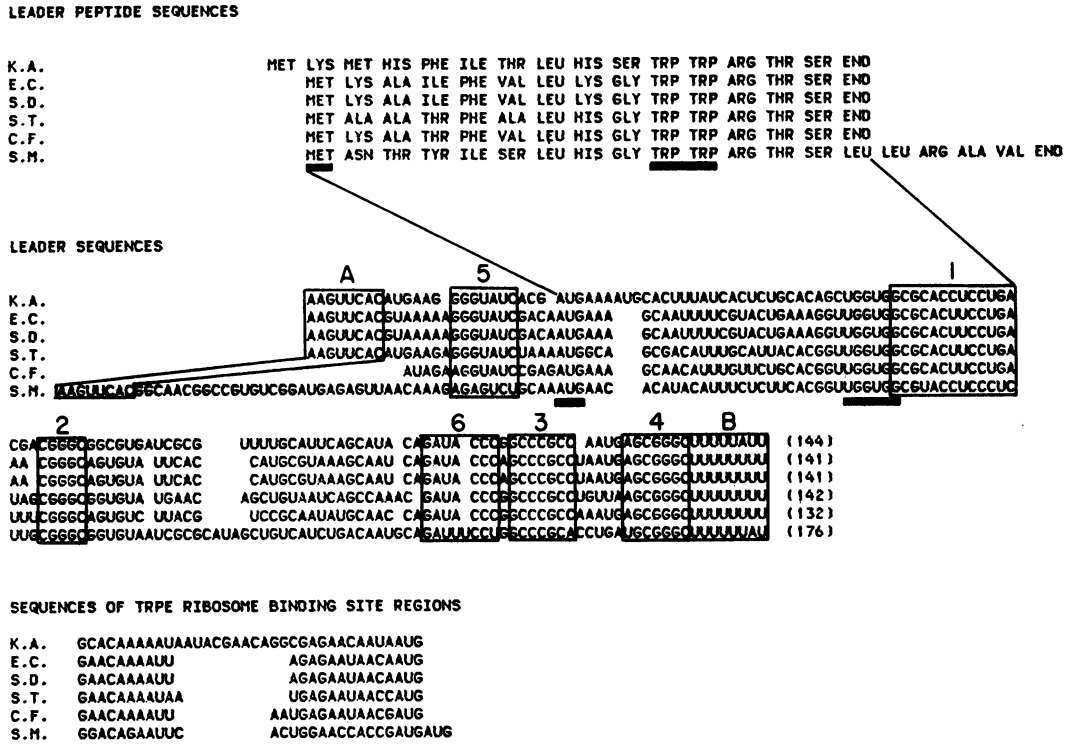

FIG. 5. Comparison of leader sequences of *K. aerogenes* (K.A.), *E. coli* (E.C.), *S. dysenteriae* (S.D.), *S. typhimurium* (S.T.), *C. freundii* (C.F.), and *S. marcescens* (S.M.). The numbers above the boxes containing highly conserved sequences correspond to the base-paired structures in Fig. 6. The bars under the sequences indicate the position of translation start sites and the tandem tryptophan residues. Note that the amino acid sequence is conserved only in the region that as RNA forms a strong secondary structure. The numbers within parentheses indicate the lengths of the individual transcripts.

rounds of synthesis of the peptide encoded by the leader transcript. Similar base pairing can be seen in transcripts of the *lamB* operon (17) and the *gal* operon (9). We presume that by the time the 5:6 base-paired stem can form, the first, decision-making ribosome would be engaged in translating the leader peptide coding region. Therefore, the 5:6 structure would not block translation initiation until the transcribing RNA polymerase molecule reached the attenuator region.

A comparison of in vitro transcription with *E. coli*, *S. typhimurium*, and *K. aerogenes trp* templates (Fig. 4) indicates that there is much

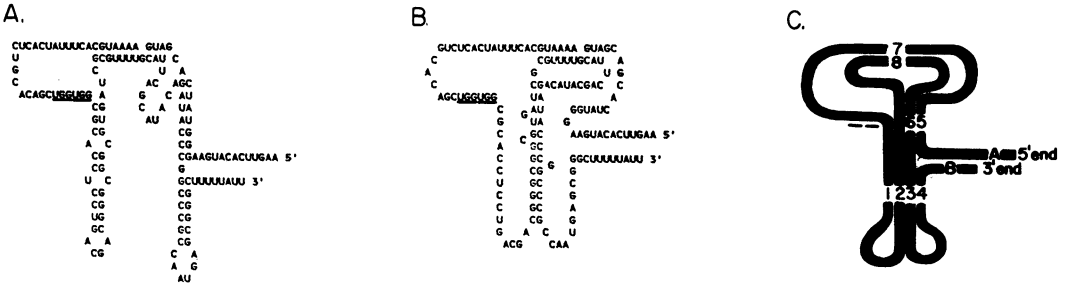

FIG. 6. Alternative secondary structures for the leader transcript. Structure A contains the 3:4 base-paired structure necessary for termination, whereas structure B contains the antiterminator structure, 2:3, which presumably forms when the translating ribosome stalls at either of the tryptophan codons. All of the possible secondary structures are presented in diagram C, including the 5:6 base-paired structure which blocks the ribosome binding site. The numbers correspond to the numbers in Fig. 5 and Table 2. Horizontal bars indicate the positions of the tandem tryptophan codons.

TABLE 2. Calculated free energy of formation of various base-paired structures

Bacterium	Calculated free energy of base-paired structure[a]				
	ΔG, 1:2	ΔG, 3:4	ΔG, 2:3	ΔG, 5:6	ΔG, 7:8
K. aerogenes	−5.1	−17.5	−27.7	−12.0	−6.6
E. coli	−6.4	−19.7	−19.3	−14.1	
Shigella dysenteriae	−6.4	−19.7	−19.3	−14.1	
S. typhimurium	−4.2	−14.5	−18.0	−12.0	
Citrobacter freundii	−10.2	−15.0	−13.0	−1.2	
S. marcescens	−17.0	−17.6	−24.2	−7.6	−9.2

[a] Calculated free energies of formation of the base paired structures depicted in Fig. 6 in kilocalories (1 kcal = 4.187 kJ) per mole for sequenced *trp* leader regions. Published procedures for calculating ΔG were used (4, 20), except that the destabilizing effect of large loops was reduced to the equivalent of a 7-nucleotide loop due to the presumed overall compactness of the leader transcript. Structures 3:4 and 2:3 are the terminator and antiterminator, respectively.

more read-through with the *K. aerogenes* template. This inefficient termination cannot be due to the instability of the terminator stem, since the *K. aerogenes* terminator stem is predicted to be as stable as in other enteric bacteria (Table 2) and is virtually identical to that of *S. typhimurium*. Comparison of the predicted stabilities of the other potential base-paired structures (Table 2) indicates that those of *K. aerogenes* are similar to those of the other enteric bacteria, except that the antiterminator structure is significantly more stable. Since 1:2 pairing in the *K. aerogenes* transcript is predicted to be weak, it is conceivable that in vitro alternative structure B, with the antiterminator (Fig. 6), may form more readily than in transcripts of the other enterics. Alternatively, the substitution of A for one of the Us at the 3′ terminus of the transcript could be responsible for the increased read-through.

The nucleotide sequence immediately beyond the site of transcription termination is the same in *K. aerogenes* as in the other enteric bacteria. However, the start codon for the *trpE* polypeptide is about 12 bp further from the end of the leader transcript terminus. The sequence immediately preceding the codon for the N-terminal methionine, the presumed ribosome binding site, is also conserved and is almost identical in all the enteric bacteria studied (Fig. 5).

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