Iron mediated methyliothiolation of tRNA as a regulator of operon expression in *Escherichia coli*

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**ABSTRACT**

*E. coli* growing in the presence of iron-binding proteins produced tRNA\textsuperscript{trp} and tRNA\textsuperscript{phe} molecules containing i\textsuperscript{6}A instead of ms\textsuperscript{2}i\textsuperscript{6}A adjacent to the anticodon. These undermodified tRNAs functioned less efficiently than the fully modified molecules when translating synthetic polynucleotides containing contiguous codons in an *in vitro* system, but did not limit the translation of MS2 RNA. We examined the possibility that the altered tRNAs with lowered translational efficiencies could relieve transcription termination at the trp and phe attenuators and lead to increased operon expression under iron restricted conditions. Using trpR mutants we found that there was indeed greater expression of the trp operon during iron restricted growth. This increase was attributable solely to the tRNA alteration induced by iron restriction.

**INTRODUCTION**

*Escherichia coli* tRNAs which contain the hypermodified nucleoside 2-methyliothio-N\textsuperscript{6}-(\Delta\textsuperscript{2}-isopentenyl)-adenosine (ms\textsuperscript{2}i\textsuperscript{6}A) show well-characterised chromatographic changes under conditions where iron is not freely available to the organism (1-4). *E. coli* growing in the presence of the iron-binding proteins transferrin, lactoferrin or ovotransferrin thus contain a population of altered tRNAs (1). It has been suggested that these altered tRNAs are involved in adapting the bacteria for growth under iron restricted conditions (1). Transfer RNAs from *E. coli* grown in chemically defined iron deficient media lack the methyliothio (ms\textsuperscript{2}) moiety of ms\textsuperscript{2}i\textsuperscript{6}A which occurs next to the 3' end of the anticodon (3). We now confirm the absence of ms\textsuperscript{2} in altered tRNA\textsuperscript{phe} and tRNA\textsuperscript{trp} from *E. coli* O111 growing normally in the presence of iron-binding proteins and present data which suggest that this physiol-
logically mediated change in post-transcriptional modification allows the tRNA molecules to function as regulatory elements in the expression of certain operons of the aromatic amino acid biosynthetic pathway.

MATERIALS AND METHODS

Organisms The bacterial strains used were: E. coli O111 K58 H2 (1); E. coli MRE 600; E. coli W3110 trpR lacZ<sub>U118</sub> As<sub>i</sub><sup>R</sup> Val<sup>R</sup>; E. coli W3110 trpR lacZ<sub>U118</sub> As<sub>i</sub><sup>R</sup> miaA; E. coli W3110 trpR lacZ<sub>U118</sub> As<sub>i</sub><sup>R</sup> miaA trp<sup>+</sup> su<sub>UCA</sub> (5); the miaA mutation was previously designated trpX (5, 6). E. coli W strains were a kind gift from Dr C. Yanofsky. All bacteria were stored at -70°C in brain heart infusion broth (BHI) containing glycerol (10%). Inocula were prepared from 3h cultures grown in BHI. Unless stated otherwise, bacteria were grown in trypticase soy broth or in trypticase soy broth containing ovotransferrin as described previously (1). Bacteria for enzyme assays were grown for 6h in trypticase soy broth supplemented with L-tryptophan (20-80 μg/ml) with or without ovotransferrin (0.5 mg/ml), or in M56 medium from which FeSO<sub>4</sub> was omitted (7); glucose (10 g/l) was employed as carbon source and the medium contained NaHCO<sub>3</sub> (0.071 M) plus Fe dicitrate (25 μM) (1), and was supplemented with casamino acids (Difco, Vitamin Assay Casamino Acids, 0.5%) and L-tryptophan (20 μg/ml).

Chromatography of <sup>3</sup>H-trp-tRNA<sub>trp</sub> <sup>3</sup>H-trp-tRNA<sub>trp</sub> was prepared by in vivo aminoacylation of E. coli tRNA and chromatographed on benzoylated-DEAE-cellulose (BD-cellulose) as described before (1). Analysis of <sup>32</sup>P, <sup>35</sup>S and <sup>14</sup>C-methyl-labelled tRNA <sup>32</sup>P-labelled tRNA was obtained from E. coli O111. The bacteria were grown first for 5h in 800 ml trypticase soy broth containing ovotransferrin as before (1), and then, for labelling (2.5h) transferred to 150 ml of a low phosphate medium equivalent to half strength trypticase soy broth made up without K<sub>2</sub>HPO<sub>4</sub> containing ovotransferrin and <sup>32</sup>P-orthophosphate (20 mCi/ 150 ml). Ovotransferrin was omitted when labelling normal tRNA. <sup>14</sup>C-Methyl-labelled tRNA was obtained by growing E. coli O111 in a methionine deficient medium containing <sup>14</sup>C-methyl-methionine (25-50 μCi/ 200 ml) (8). Methionine deficient medium (Difco, 5%) was made up with water and contained NaHCO<sub>3</sub> and ovotransferrin (1); the bacteria were grown
at 37°C under 5% CO₂-95% air (1). Again ovotransferrin was omitted when labelling tRNA from iron replete organisms. Inocula were grown in the same medium containing unlabelled L-methionine (0.01 mg/ml). *E. coli* Olll tRNA was labelled with ³⁵S using ³⁵SO₄ (10 Ci/ 200 ml) as described by Brownlee (8); when labelling altered tRNA the medium also contained ovotransferrin and NaHCO₃ and the gas phase was 5% CO₂-95% air.

Labelled tRNA<sub>trp</sub> was purified by a procedure similar to that described by Joseph and Muench (9). Bulk tRNA was isolated as before (1) and deacylated. tRNA<sub>trp</sub> was aminoacylated in vitro with L-(G⁻³H)-tryptophan (6 Ci/mmol) and the ³H-trp-tRNA<sub>trp</sub> isolated by BD-cellulose chromatography. The labelled tRNA<sub>trp</sub> was then deacylated and re-chromatographed on the same column. Both normal and altered tRNA<sub>trp</sub> eluted ahead of the corresponding aminoacylated species during chromatography on BD-cellulose using an ethanol gradient (0-15%, 600 ml) (1). Unfractionated aminoacyl-tRNA synthetases were prepared from *E. coli* MRE 600 and subjected to DEAE-cellulose chromatography before use (10). Normal and altered tRNA<sub>trp</sub> species were routinely renatured before charging with tryptophan by heating at 50°C for 10 min in 0.1 M tris-HCl buffer (pH 7.8), 0.01 M Mg acetate (11,12). Uncharged labelled tRNA<sub>phe</sub> was recovered from the salt wash fraction obtained during the isolation of trp-tRNA<sub>trp</sub> on BD-cellulose. It was aminoacylated with ³H-phenylalanine (19 Ci/mmol) and chromatographed again on BD-cellulose where it was eluted with an ethanol gradient (1). The peak of phe-tRNA<sub>phe</sub> was used as source of tRNA<sub>phe</sub> for nucleotide analysis of ³²P- and ¹⁴C-methyl labelled tRNA<sub>phe</sub>. ³⁵S-labelled phe-tRNA<sub>phe</sub> was purified further; it was deacylated and rechromatographed on BD-cellulose using a salt gradient (0.4 -1.5 M NaCl in 0.05 M Na acetate, 0.01 M Mg acetate, pH 4.5; 600 ml).

Purified labelled tRNAs were digested with ribonuclease-T₂ (13) and the resulting nucleotide mixture examined. Digests of ³²P-tRNA were analysed for ¹⁶Ap and ms²¹⁶Ap using chromatography systems 'a' and 'b' of Brownlee (8) and by electrophoresis on DEAE-cellulose paper (Whatman DE-81) at pH 3.5 (0.5% pyridine-0.5% acetic acid)(8). Methylated nucleotides from ¹⁴C-methyl labelled tRNAs were characterised by electrophoresis on
Whatman 3MM paper at pH 3.5 (4 KV) and by paper chromatography using isopropanol-HCl (system 'a') (8). $^{35}$S-nucleotides from digests of $^{35}$S-tRNA were characterised by electrophoresis at pH 3.5 (Whatman 3MM paper (8)). Ap,Cp,Gp,Up and s$^4$Up (PL Biochemicals Inc., USA) were used as markers. Radioactive compounds were obtained from The Radiochemical Centre, Amersham.

**Polypeptide synthesis in vitro**

_E. coli_ was grown in trypti-case soy broth with or without ovotransferrin from an inoculum of $10^6$ cell/ml for 4-5 h in 5-15 l cultures. S30 extracts were prepared in 10 mM tris-HCl buffer (pH 7.8), 10 mM Mg acetate, 60 mM NH$_4$Cl, 6 mM β-mercaptoethanol (14). S160 supernatants and ribosomes were obtained from S30 extracts of iron-replete organisms; ribosomes were washed twice before storing (14). The concentration of ribosomes was determined using A$_{260}$ (14). S160 supernatants were freed from tRNA by DEAE-cellulose treatment (15). All protein synthesis components and extracts were stored at -70°C.

Unfractionated tRNA containing the normal or altered tRNA species was prepared from _E. coli_ O111 grown in broth or in broth containing ovotransferrin (1). tRNA was isolated in the usual way and treated with DEAE-cellulose before dialysing and freeze drying (16). Unfractionated tRNA from iron-restricted bacteria accepted 15% more phenylalanine per A$_{260}$ unit than that from the iron replete bacteria.

For translating synthetic polynucleotides, the incubation mixture contained: 15 mM Mg acetate, 60 mM tris-HCl buffer (pH 7.8), 6 mM β-mercaptoethanol, 1.6 mM ATP, 0.15 mM GTP, 2.8 mM phosphoenolpyruvate and 20 µg/ml pyruvate kinase. The incubation mixture also contained either 30 A$_{260}$ units/ml of an S30 extract (all extracts had A$_{260}$/A$_{280}$ ratios of between 1.83 and 1.9) or, in tRNA dependent assays, 600 µg/ml S160 supernatant proteins, 260 µg/ml ribosomes; poly U or poly UC (U:C 1.3:1, Miles Biochemicals) was present at 200 µg/ml, unlabelled L-phenylalanine was 40 µM and $^3$H-phenylalanine (17 Ci/mmol) was at 20 uCi/ml. When translating MS2 RNA (12 A$_{260}$ units/ml, Miles Biochemicals) the incubation mixture was the same as above except that the Mg concentration was reduced to 10 mM and all 20 amino acids (40 µM each) were present. Leucovorin (120 µM, Serva) was also added and polypeptide synthesis assayed by the incorporation of $^{14}$C-valine.
(285 mCi/mm; 6.7 μCi/ml). Incubations were carried out at 37°C and aliquots assayed for hot trichloroacetic acid precipitable radioactivity. The radioactivity incorporated was corrected for backgrounds obtained in the absence of added mRNA. Radiochemicals were from The Radiochemical Centre, Amersham.

Enzyme assays  Anthranilate synthase activity in cell extracts was measured by following anthranilate formation fluorimetrically (17). Extracts, prepared by sonication, were dialysed for 2h against phosphate buffer (0.1 M, pH 7.6) containing β-mercaptoethanol (1 mM) before use. Assays were carried out at ambient temperature (22-23°C); one unit of activity represents the formation of 1 µmol of anthranilate/min. Tryptophan synthase activity in cell extracts was measured by the method of Smith and Yanofsky (18). One unit of activity was taken as the amount that catalysed the conversion of 0.1 µmol indole to tryptophan at 37°C in 20 min. Protein concentrations were determined by the method of Lowry et al (19).

RESULTS AND DISCUSSION

tRNA alterations under iron restriction  When E. coli O111, an organism often associated with neonatal diarrhoea, is grown in natural secretions or in defined media containing ovotransferrin, 90-95% of tRNA^phe, tRNA^tyr and tRNA^trp appear as chromatographically altered species (1, and Fig la). The presence of i^6A instead of ms^2i^6A in the altered tRNAs has been inferred previously from the major shift in their elution position on BD-cellulose (1) and on RPC-5 chromatography (unpublished data), and their rapid conversion to the normal species on adding Fe^3+ (1). This view is now supported by results which show that the chromatographic mobility of trp-tRNA^trp lacking both the methylthio (ms^2) and isopentenyl (i^6) modification is unaffected by iron-binding proteins in the growth medium (Fig lc) and elutes ahead of trp-tRNA^trp thought to lack only the ms^2 moiety (Figs la,lb). tRNA^trp lacking both the ms^2 and the i^6 groups was isolated from E. coli W3110 trpR miaA (5); tRNA^trp from miaA mutants has an unmodified adenosine adjacent to the 3' end of the anticodon (6,20). The parent strain, E. coli W3110 trpR, responded normally to iron restriction (Fig lb) and, like E. coli O111 (Fig la),
Figure 1  Influence of hypermodification on the chromatographic mobility of $^3$H-trp-tRNA$^{trp}$ extracted from bacteria grown in: (●) trypticase soy broth and (○) trypticase soy broth containing ovotransferrin.  a) E. coli O111 ; b) E. coli W3110 trpR ; c) E. coli W3110 trpR miaA . In a) and b) the BD-cellulose column was developed with a 0-15% ethanol gradient (600 ml) and in c) with a 0-10% ethanol gradient (600 ml) in the same buffer (1). When c) was developed with the 0-15% ethanol gradient, the peaks eluted together a little earlier than shown above, but were slightly skewed.
contained altered trp-tRNA\textsubscript{trp} when grown in the presence of ovo-
transferrin. Similar relative chromatographic mobilities have
been shown for three forms of suppressor tRNA\textsubscript{tyr\textsuperscript{UAG}}, the fully
modified molecule containing ms\textsuperscript{2}i\textsuperscript{6}A, the molecule lacking ms\textsuperscript{2}
and that lacking both the ms\textsuperscript{2} and the i\textsuperscript{6} modification of A\textsuperscript{37}(21).
The absence of the ms\textsuperscript{2} group and the presence of i\textsuperscript{6}A in altered
tRNA\textsubscript{trp} and tRNA\textsubscript{phe} from \textit{E. coli} O\textsubscript{111} grown in the presence of
ovotransferrin was confirmed by analysis of \textsuperscript{32}P-, \textsuperscript{35}S- and \textsuperscript{14}C-
methyl labelled tRNAs (Table 1); all other nucleosides containing
a methyl and/or sulphur residue (22) were still present.
Our results are consistent with the view that iron restriction
results in a specific under-modification of all \textit{E. coli} tRNAs
which usually contain ms\textsuperscript{2}i\textsuperscript{6}A to i\textsuperscript{6}A containing species (1,3).

\textbf{Functional activity of altered tRNAs} Earlier work had shown
that a ms\textsuperscript{2}-deficient suppressor tRNA\textsubscript{tyr\textsuperscript{UAG}} isolated from O\textsubscript{80} su\textsubscript{3}
phage infected \textit{E. coli} was about 50\% as efficient as the fully
modified tRNA in suppressing UAG \textit{in vitro} (21). This tRNA also
showed a marked reduction in its ability to bind to ribosomes in
response to UAG. Undermodified \textit{E. coli} tRNA\textsubscript{phe} lacking ms\textsuperscript{2} and
other modifications, however, behaved normally in ribosom binding

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
 & tRNA\textsubscript{trp} & & & tRNA\textsubscript{phe} \\
 & +Fe\textsuperscript{3+} & -Fe\textsuperscript{3+} & +Fe\textsuperscript{3+} & -Fe\textsuperscript{3+} \\
\hline
ms\textsuperscript{2}i\textsuperscript{6}A & + & - & + & - \\
i\textsuperscript{6}A & - & + & - & + \\
S\textsuperscript{4}U & + & + & + & + \\
rT & + & + & + & + \\
m\textsuperscript{7}G & + & + & + & + \\
Cm & + & + & - & - \\
\hline
\end{tabular}
\caption{Methylated and thiolated nucleotides in tRNA\textsubscript{trp}
and tRNA\textsubscript{phe} from \textit{E. coli} O\textsubscript{111}}
\end{table}

( +Fe\textsuperscript{3+}): bacteria grown in trypticase soy broth
(-Fe\textsuperscript{3+}): bacteria grown in trypticase soy broth
with ovotransferrin.
experiments (23, 24) although a similar, but not identical, undermodified tRNA\textsubscript{phe} also lacking ms\textsuperscript{2}, was 60% as efficient as normal tRNA\textsubscript{phe} in supporting poly-U directed protein synthesis (25, 26). Bacillus subtilis tRNA\textsubscript{phe} lacking ms\textsuperscript{2} and containing an unmodified G instead of Gm in the first position of the anticodon also supported peptide bond formation less efficiently than the fully modified tRNA\textsubscript{phe} (27). In view of these somewhat conflicting results we examined the ability of tRNA lacking ms\textsuperscript{2} from E. coli O111 grown under iron restricted conditions to function in polypeptide synthesis.

When the kinetics of aminoacylation were examined, we found that both altered tRNA\textsubscript{phe} and tRNA\textsubscript{trp} were charged at a rate similar to that of the normal tRNAs. This is consistent with previous findings (21, 28, 29). A reduced rate of aminoacylation is usually associated with the absence of additional modified bases in the tRNA molecule. For example, E. coli tRNA\textsubscript{phe} lacking ms\textsuperscript{2} and several other modified residues is aminoacylated at a considerably slower rate than the normal tRNA\textsubscript{phe} (25, 26). Altered tRNA\textsubscript{trp} from iron-restricted E. coli O111 was also found to behave normally with respect to the formation of the inactive conformer (11, 12). Although the loss of the ms\textsuperscript{2} group had no effect on aminoacylation we found that it did alter the ability of the tRNA to function in polypeptide synthesis. Altered tRNA\textsubscript{phe} from E. coli O111 grown in the presence of ovotransferrin, and lacking ms\textsuperscript{2}, performed poorly in a protein synthesising system directed by poly-U. Figure 2 shows that it had only 20–30\% efficiency of the normal methylthiolated tRNA\textsubscript{phe} from iron replete bacteria. Altered tRNA\textsubscript{phe} translated poly-UC with 40–50\% efficiency of the normal tRNA (not shown). The slightly better performance in reading poly-UC may mean that the ability of altered tRNA\textsubscript{phe} to read codon UUU is reduced more than its ability to read UUC. Preliminary results showed that altered tRNA\textsubscript{trp} also had a reduced translational capacity when translating poly-UG.

Unlike most natural mRNAs, these synthetic polynucleotides contain long repeats of the same codons and the translation of such contiguous codons might therefore accentuate the difference between the reading efficiency of normal and altered tRNAs. The
Figure 2  Translation of poly-U by normal and altered tRNA. Polyphenylalanine synthesis with normal tRNA (●) and with ms<sup>2</sup>-deficient tRNA (○). The results are expressed as a percentage of the incorporation achieved in the 40th minute with the normal (ms<sup>2</sup>16A) tRNA preparation. This corresponded to 2300 cpm per 50 µl aliquot. S160 supernatants and ribosomes were from iron-replete organisms.

Fact that *E. coli* grew well in the presence of iron-binding proteins suggested that the lack of ms<sup>2</sup> in the tRNAs had little overall effect on protein synthesis (1). A similar conclusion regarding the effect of the lack of hypermodification of A37 in tRNA on general protein synthesis was reached by Eisenberg *et al* (6) who found little difference in doubling time between *E. coli miaA* and the isogenic wild-type parent. We therefore examined the effect of the altered tRNA population induced by iron restriction on the translation of natural mRNA using MS2 phage RNA as the message. Cell free extracts from *E. coli* Olll, however, translated MS2 RNA poorly and to overcome this difficulty we used cell extracts from *E. coli* MRE 600. BD-cellulose chromatography of trp-tRNA<sub>trp</sub> and phe-tRNA<sub>phe</sub> confirmed that the chromatographically distinct altered tRNAs were generated by growth of *E. coli* MRE 600 in the presence of ovotransferrin; 90-95% of tRNA<sub>phe</sub> and tRNA<sub>trp</sub> were present as the under-modified species. In addition, polypeptide synthesis directed by poly-U using S30 extracts of *E. coli* MRE 600 grown in broth containing ovotransferrin was only about 20% as efficient as that using S30 extracts of the bacteria grown in broth alone.
When the S30 extracts from broth-grown \textit{E. coli} MRE 600 was programmed with MS2 RNA, 36 \( \mu \) moles valine were incorporated per 150 \( \mu \)l reaction mixture after 30 min. The S30 extract from the bacteria grown in broth containing ovotransferrin, however, incorporated nearly twice this amount, 70 \( \mu \) moles per 150 \( \mu \)l volume. The results were consistent in that extracts from the ovotransferrin grown cells were always slightly more active with MS2 RNA than those from the iron replete bacteria. This might be due to differences in initiation or elongation factor level between the two extracts. Clearly, however, the S30 extracts from the bacteria grown in the presence of ovotransferrin, although translating poly-U poorly, did not have a reduced overall translational capacity. This was confirmed by using a tRNA dependent system from MRE 600 to translate natural mRNA. No difference was found between the ability of the unfractonated tRNA containing \( \text{ms}^2 \)-deficient molecules and the tRNA containing fully modified species to translate MS2 RNA. MS2 RNA stimulated the incorporation of valine 70 fold with both tRNA preparations (5 A260 units/ml) to give an incorporation of 26 \( \mu \) moles valine per 150 \( \mu \)l reaction volume after 25 min. In the MRE 600 derived system, \( \text{ms}^2 \)-deficient tRNA\(^{\text{phe}} \) supported poly-U directed poly-peptide synthesis with 60% efficiency of the fully modified species (15 mM Mg acetate; tRNA 5 A260 units/ml; poly-U 200 \( \mu \)g/ml).

\textbf{Methylthiolation of tRNA and regulation of the tryptophan operon}

Our results, limited as they may be by the constraints of an \textit{in vitro} translating system, showed that differences between the translational efficiencies of \( ^6\text{A-tRNAs} \) and \( \text{ms}^{2}_6\text{A-tRNAs} \) were detected when the tRNAs read synthetic polynucleotides containing regions of contiguous codons. We could not detect any limitation in the translation of MS2 RNA by unfractonated tRNA containing the altered molecules (\( ^6\text{A-tRNAs} \)) instead of the fully modified species (\( \text{ms}^{2}_6\text{A-tRNAs} \)). This may be partly due to the fact that only six species of tRNAs contain this particular modification. It might be thought, therefore, that the appearance of the undermodified tRNA population in the bacterial cell during iron restricted growth would have little metabolic consequence. However, repeating codon sequences for phenylalanine...
and tryptophan have been found in the leader transcripts of the
phenylalanine (phe) and tryptophan (trp) operons (30,31), and
the rate of their translation is believed to be an important
factor in regulating transcription termination at the attenuator
of these operons (32,33). In the trp and phe leader RNAs a coding
region containing contiguous codons which correspond to the
amino acid that regulates the operon precedes the terminator
site (30,31). Regulation of transcription appears to depend upon
the formation of mutually exclusive RNA secondary structures
which are in turn determined by the progress of ribosomes trans-
slating the RNA (33). Because of the adjacent codons, the rate of
translation is sensitive to the concentration of the respective
amino acid through available levels of charged tRNAs. Thus,
structural gene expression varies in response to the level of
charged tRNA present, a plentiful supply ensuring rapid read-
through of the leader peptide coding region and signalling ter-
mination of transcription. The reduced translational efficiencies
of tRNA\textsubscript{trp} and tRNA\textsubscript{phe} lacking the ms\textsuperscript{2} group when translating
the contiguous codons in synthetic polynucleotides suggested to
us that these physiologically altered tRNAs, should they also
operate less efficiently in vivo, might function to relieve
transcription termination and lead to a greater expression of the
operons under iron restricted conditions. Relaxation of trans-
scription termination at the trp and phe attenuators has been
shown to occur constitutively in E. coli carrying the miaA
mutation. In these mutants, where tRNA\textsubscript{trp} and tRNA\textsubscript{phe} have an un-
modified A37, the structural genes of the trp and phe operons
are expressed at a level greater than that achieved by de-
repression alone (5,31). This, it has been suggested, is due to
inefficient translation of the regulatory codons by ms\textsuperscript{2,6}-deficient
tRNA\textsubscript{trp} and tRNA\textsubscript{phe} (6). Elevated levels of enzymes from
the trp and other operons of the aromatic amino acid biosyn-
thetic pathway have also been detected in iron starved E. coli (34).
Although much of this increase is undoubtedly due to derepression
of the operons through depletion of the aromatic amino acids
during iron restriction, a residual portion cannot be explained
be derepression alone (34).

To determine the possible participation of ms\textsuperscript{2}-
deficient tRNA$^{\text{trp}}$ generated by iron restriction in regulating transcription termination at the trp attenuator, we measured anthranilate synthase and tryptophan synthase activities in cell extracts of *E. coli* W3110 trpR grown in broth and in broth containing ovotransferrin; in trpR strains the trp operon is constitutively derepressed and allows regulation at the trp attenuator to be examined in the absence of the dominant repression control (5). Results showed that anthranilate synthase activity was elevated 2-3 fold in the iron restricted bacteria (Table 2). A similar increase was found in the specific activity of tryptophan synthase in bacteria grown in broth containing ovotransferrin (not shown), strongly suggesting that the presence of ms$^2$-deficient tRNA$^{\text{trp}}$ in the cells was indeed relieving transcription termination at the trp attenuator. The transcription of the trp operon is, however, known to be influenced by the growth rate of *E. coli* (35, 36). We therefore examined the possibility that the increased expression of this operon was due to the slightly longer doubling time.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth Medium (d)</th>
<th>Anthranilate Synthase Specific Activity (units/ mg)</th>
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<tbody>
<tr>
<td><em>E. coli</em> W3110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>trpR</td>
<td>Broth (26)</td>
<td>2.4 ± 0.3(7)</td>
</tr>
<tr>
<td>trpR</td>
<td>Broth + ovotransferrin (36)</td>
<td>6.1 ± 0.5(5)</td>
</tr>
<tr>
<td>trpR</td>
<td>M56 + Casamino acids (40)</td>
<td>2.6 ± 0.4(3)</td>
</tr>
<tr>
<td>trpR miaA</td>
<td>Broth (33)</td>
<td>16.3 ± 4.0(4)</td>
</tr>
<tr>
<td>trpR miaA</td>
<td>Broth + ovotransferrin (33)</td>
<td>15.7 ± 1.8(3)</td>
</tr>
<tr>
<td>trpR miaA trpT su$^+$ UGA</td>
<td>Broth (28)</td>
<td>4.5 ± 0.6(3)</td>
</tr>
<tr>
<td>trpR miaA trpT su$^+$ UGA</td>
<td>Broth + ovotransferrin (40)</td>
<td>4.4 ± 1.0(5)</td>
</tr>
</tbody>
</table>

Specific activity: the numbers in parentheses indicate the number of independent cultures of each type that were assayed for anthranilate synthase activity.

d: doubling time in minutes.
of the bacteria in the iron restricted medium; *E. coli* W3110 trpR had a doubling time of 36 min in broth containing ovotransferrin and 26 min in broth alone. By growing *E. coli* W3110 trpR in an iron-replete minimal medium containing casamino acids we obtained a growth rate similar to that of the bacteria growing in broth plus ovotransferrin; the doubling time was 40 min. Results showed that changes in growth rate within this range had no effect on anthranilate synthase activity (Table 2). To examine the possibility that iron restriction was affecting the expression of the trp operon in some way unconnected with the alteration in tRNA\text{trp}, we used mutants in which the state of the tRNA is unaffected by the availability of iron in the medium. We compared the activity of anthranilate synthase in *E. coli* W3110 trpR miaA grown in broth with that in the same mutant grown in broth containing ovotransferrin. Results showed little difference between them (Table 2). As expected the miaA mutation led to a higher normal level of trp operon expression due to constitutive relief of transcription termination at the attenuator (5,6). Similarly, we found no difference between the activity of anthranilate synthase in *E. coli* W3110 trpR miaA trpT su\textsuperscript{+} UGA grown in broth and that in this mutant grown in broth containing ovotransferrin (Table 2); in both cases the presence of UGA suppressor tRNA\text{trp} largely overcame the miaA phenotype. Replacement of wild type tRNA\text{trp} by the suppressor tRNA\text{trp} allows efficient transcription termination to occur at the trp attenuator, resulting in a much reduced level of anthranilate synthase activity, even though the tRNA still lacks both the i\textsuperscript{6} and ms\textsuperscript{2} modifications due to the miaA mutation (5,6). This is believed to be due to the unusual translational property of this suppressor tRNA (6).

From these results we conclude that the increased expression of the trp operon in *E. coli* grown in the presence of iron-binding proteins is due to decreased transcription termination at the attenuator caused by the presence of ms\textsuperscript{2}-deficient tRNA\text{trp} in the cell. This effect is less than that produced when tRNA\text{trp} lacks both ms\textsuperscript{2} and i\textsuperscript{6} (miaA cells), a situation which might have been predicted from knowledge of the relative effect of losing ms\textsuperscript{2} alone and of losing both ms\textsuperscript{2} and i\textsuperscript{6} on the translational activity of tRNA\textsubscript{TYR UAG} (21).
Our results, taken together with those of others suggest that relaxation of attenuation in the trp and phe operons in E. coli can be regulated physiologically not only by the concentration of the respective amino acid through levels of charged tRNAs, but also by an iron related change in the tRNA molecules themselves. Although we have not tested the idea, a combination of these two effects might provide a sensitive way of amplifying each individual signal. We think that such regulatory features in these, and possibly other (37) operons of the aromatic amino acid biosynthetic pathway may be involved with adapting E. coli for growth in iron restricted environments and especially for adapting pathogenic strains for growth in mammalian body fluids which contain iron-binding proteins (1,38). During iron restriction, E. coli synthesises and secretes the iron chelator enterochelin, which removes iron from iron-binding proteins, or from insoluble ferric complexes, and transports it to the bacterial cell (38-41). Enterochelin is synthesised from chorismic acid by way of a branch of the aromatic amino acid biosynthetic pathway (39). De-repression of the enterochelin system during iron restriction will require adjustments to the whole aromatic pathway to ensure production of sufficient chorismic acid and continued production of the amino acids and other aromatic compounds, if required. Iron itself is also essential for the biosynthesis of all aromatic compounds in E. coli; it is a component of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, the first enzyme in the common pathway (42). The ability to control the expression of various operons both through the level of charging of tRNA and by the degree of modification of the tRNA, in turn dependent upon iron levels, may well provide the cell with considerable regulatory flexibility.

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2623