Alteration of transfer ribonucleic acid of *Neisseria meningitidis* during growth in the presence of human transferrin

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Summary. Iron-related tRNA alterations have been shown to occur in several pathogens but nothing has been reported about the effect of iron on meningococcal tRNA. The chromatographic elution profile of ³H-tryptophan-tRNA^{trp} from a *Neisseria meningitidis* strain grown under different conditions was examined. The profile showed an early (P1) and a late (P2) eluting species of tRNA^{trp}, but the proportion of the two species varied under different growth conditions. The elution profile of trp-tRNA^{trp} from bacteria grown in iron-sufficient Mueller Hinton broth yielded a minor P1 species and a major P2 species, whereas under iron-restricted growth induced by desferrioxamine, the pattern was one of a major P1 species and minor P2 species. Iron-restriction induced by human transferrin (HTF) resulted in almost equal amounts of the two tRNA^{trp} species (P1 ≈ P2). Differences in the proportions of the tRNA species were also found between cells grown in liquid medium (P1 < P2) and on the same medium solidified with agar (P1 > P2). The growth phase of the bacteria did not have any effect on the tRNA^{trp} elution profile. Changes in tRNA^{trp} induced by HTF were readily and completely reversible when the cells were transferred to an iron-rich medium, but those induced by desferrioxamine remained irreversible for a long period (16 h) after such transfer.

Introduction

There is now overwhelming evidence that many crucial virulence determinants of pathogenic bacteria are environmentally regulated and expressed only under certain conditions.¹ Virulence genes are regulated by various environmental signals including temperature, osmotic pressure, oxygen and the availability of essential nutrients, such as iron. The restricted availability of iron in tissue fluids not only presents microbial pathogens with the problem of acquiring sufficient for multiplication *in vivo*, but also constitutes a major signal which co-ordinately regulates the expression of a number of virulence and metabolic genes.^{1,2}

Much of our understanding of how organisms adjust *in vivo* to iron limitation comes from studies on *Escherichia coli*. During iron restriction induced by host iron-binding proteins, *E. coli* produces siderophores and expresses a number of new outer-membrane proteins.² The expression of many of the iron-

regulated genes is negatively controlled by a global repressor protein (Fur) which uses ferrous iron as a co-repressor.³ An additional global regulatory system based on under-modification of several transfer ribonucleic acids (tRNAs) also appears to operate.² Iron restriction in *E. coli* produces a family of specifically altered tRNAs. Thus tRNAs for phenylalanine, tyrosine, tryptophan, serine, cysteine and leucine in *E. coli* grown under iron restriction lack the methylthio group (ms²) present on the isopentenyl-adenosine (i⁶A) located next to the anticodon at position 37 (A37) in tRNA from iron-replete cells. These under-modified forms appear on chromatography as early eluting tRNA species and, on adding iron,⁴ are converted rapidly (40 min) to the fully modified late-eluting species. The same iron-related tRNA alteration has been shown to occur also in *E. coli* recovered directly from the peritoneal cavities of infected animals.⁵ Under-modification of the nucleotide at A37 in tRNA induces strong pleiotropic effects on cell physiology; in some instances by affecting the translational efficiency of the tRNA, in others by altering codon context effects, and in others by yet unknown mechanisms.^{6–12} This modification may also lead to an increase in the frequency of spontaneous mutations when cells need to adapt to environmental stress.^{13,14}

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Fig. 1. Growth of *N. meningitidis* strain SD (B15 P1.16) under different conditions: MHB (●); MHB-TF (○); MHB-D (■); transferred from MHB-D at 6 h to MHB-D with added ferric di-citrate (▲), or to fresh MHB (△).

Fig. 2. Western blot of total outer-membrane proteins of *N. meningitidis* strain SD, (B15 P1.16) grown in: 1, MHB; 2, MHB-D; 3, MHB-TF for 24 h; 4, in MHB-TF for 6 h. All membranes were exposed to HRP-HTF overnight and developed before probing with rabbit anti-FrpB (AR-70) antiserum. FrpB was then visualised with peroxidase conjugated goat anti-rabbit (BioRad); *TBP-2, **FrpB.

Although much less is known about the molecular mechanisms of iron regulation in other organisms, *fur*-like systems have been found in pathogens such as *Vibrio cholerae*,¹⁵ *Corynebacterium diphtheriae*¹⁶ and

Yersinia pestis,¹⁷ and tRNA changes have been seen in *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* when grown in the presence of an iron-binding protein.¹⁸ It is already known that *Neisseria meningitidis* expresses several new outer-membrane proteins during growth under iron limitation. Some of these proteins are believed to be important for virulence and might be useful protective antigens.^{19–21} Nothing has been reported about the effect of iron restriction on meningococcal tRNA. In the work reported here, the effect of different growth conditions on the chromatographic behaviour of tRNA from *N. meningitidis* was examined.

Materials and methods

Bacterial growth conditions

N. meningitidis strain SD (B15 P1.16), also called strain 70942,²² was grown in Mueller Hinton Broth (MHB) or on Mueller Hinton Agar (MHA) (Oxoid). For iron limitation, desferrioxamine (Ciba) was added to the broth or agar to 25 μ M, yielding MHB-D and MHA-D respectively, as described previously.^{19, 23} Broth cultures were incubated overnight at 37 °C in CO₂ 5% in flasks with shaking at 80 rpm. In some experiments, bacteria were grown overnight in MHB or MHB-D, collected by centrifugation at 1200 *g*, transferred to fresh MHB or MHB-D, and incubated for a further period.

Other iron chelators used in broth cultures were

Fig. 3. BD-cellulose chromatography of [^3H]-trp-tRNA^{trp} extracted from *N. meningitidis* strain SD (B15 P1.16) grown in MHB (.....) and in MHB-D (—) following overnight incubation. Ethanol gradient (-----).

ethylenediaminedihydroxyphenylacetic acid (EDDA, Sigma) 14 $\mu\text{g}/\text{ml}$, yielding MHB-E, and human transferrin (HTF, Sigma) 1 mg/ml , dissolved in NaHCO_3 0.6% before adding to MHB, yielding MHB-TF. Ferric di-citrate, prepared as described previously,²⁴ was used as an iron supplement at concentrations (v/v) of 0.5% for MHB and MHA and 1% for MHB-D and MHA-D.

Comparative growth curve

MHB, MHB-D and MHB-TF were inoculated with an overnight static phase MHB culture of strain SD to give 10^5 cfu/ml and incubated with shaking as above. The viable count of the cultures was determined every hour by the micro-assay procedure described previously.²⁵ After 6 h, the MHB-D culture was divided into three equal volumes: one volume was re-incubated without change; another was supplemented with ferric di-citrate 1% v/v before re-incubation; and the third volume was centrifuged at 1200 g for 20 min at 4°C and the harvested cells were resuspended in fresh MHB and then re-incubated. While this sample was being centrifuged, all the other samples were removed from the incubator and stored temporarily at 4°C before re-incubation at 37°C.

Aminoacylation, extraction and chromatography of aminoacyl-tRNA

Transfer RNA was aminoacylated *in vivo* with a 10-min pulse of ^3H -labelled amino acids as described previously⁴ with the minor modifications detailed below. Cells were harvested from broth by centrifugation at 1200 g , and washed with and resuspended in 10 ml of glucose-bicarbonate buffer (glucose 0.1% w/v, 0.15 M NaCl and 0.01 M NaHCO_3 , pH 7.6) at 37°C; 100 μCi L-[G- ^3H]-tryptophan (Amersham International plc) was added, the mixture was incubated for 10 min at 37°C and cells were harvested by centrifugation (1200 g at 4°C for 30 min). The cells were washed once with solution A (0.01 M magnesium acetate, 0.05 M sodium acetate and 0.15 M NaCl, pH 4.5) and resuspended in 2 ml of solution A. Carrier cells (1–2 g of *E. coli* strain O111) were mixed with the labelled cells and the volume was made up to 10 ml with solution A. An equal volume of phenol 80% w/v was added to the cell suspension and the mixture was shaken at 4°C for 20 min. The aminoacyl-tRNA was precipitated from the aqueous phase by potassium acetate (pH 4.5, 20% w/v) and ethanol and re-precipitated from solution B (0.01 M magnesium acetate, 0.05 M sodium acetate and 1 M NaCl, pH 4.5); the

final ethanol precipitate was dissolved in 5 ml of solution A for chromatography.

A benzoylated DEAE-cellulose (BD-cellulose, Cellex-BD, BioRad) column was packed as described before⁴ and equilibrated with solution B. After the addition of 10 ml of solution A, the aminoacyl-tRNA preparation was applied. The column was washed with 100 ml of solution B (flow rate 0.5 ml/min) and bound aminoacyl-tRNA was eluted with a linear gradient of ethanol (0–20% v/v) in solution B; 5-ml fractions were collected and the radioactivity present was measured as described previously.⁴ Elution profiles were obtained by plotting the amount of radioactivity found in each fraction, expressed as a percentage of the total radioactivity recovered, *versus* the fraction number.

Extraction of outer-membrane proteins (OMPs), SDS-PAGE and Western blotting

OMPs were extracted from *N. meningitidis* strain SD, separated by SDS-PAGE and Western blotted as described previously.^{19,23} Polyclonal monospecific antibodies (AR-70) against the 70-kDa iron-regulated protein of meningococci (now referred to as FrpB) were raised, and used in Western blots as described previously.²³ Horseradish peroxidase-labelled HTF (HRP-HTF) was also used as described²⁶ to visualise transferrin binding protein, TBP-2 on Western blots.

Results

Growth of *N. meningitidis* under different conditions

The effects of various iron chelators on the growth of *N. meningitidis* in MHB are shown in fig. 1. The bacteria remained in a lag phase for 2 h in MHB, 3 h in MHB-TF and 4 h in MHB-D before exponential growth started. The exponential growth rate in MHB-TF was slightly slower than that in MHB and was extremely slow in the desferrioxamine-containing medium (MHB-D). The expression of the iron-regulated 70-kDa protein (FrpB) and the HTF-binding protein TBP-2 by *N. meningitidis* strain SD when grown in MHB-D and MHB-TF confirmed that the organisms were growing under nutritional stress (fig. 2). These proteins were not expressed when the strain was grown in MHB or on MHA.

The deleterious effect of desferrioxamine on growth rate was swiftly and almost completely reversed as soon as ferric di-citrate was added to the medium or the cells were transferred to fresh MHB. Meningococci did not grow well in the presence of EDDA (MHB-E) although they expressed the iron regulated proteins.

Alteration in tRNA^{trp} profiles induced by various growth conditions

The chromatographic elution profile of ³H-trp-

tRNA^{trp} from overnight MHB cultures of *N. meningitidis* is shown in fig. 3; c. 23% of the tRNA^{trp} eluted between 2% and 5.5% ethanol, yielding a minor species peak (P1) and was followed by a major species peak (P2) (71%) eluting between 5.5% and 8.5% ethanol. This pattern of a minor P1 followed by major P2 was reproducible in repeat experiments and was the same whether or not the cells were in the lag, exponential or stationary phase of growth at the time of tRNA extraction. In contrast, chromatographic analysis of tRNA^{trp} from cells grown overnight in MHB-D produced an elution profile which was essentially the reverse of that produced under iron-sufficient conditions, with a major species (72%) peak eluting early at the P1 position and a minor species (21%) peak late at the P2 position. The switch from a

Fig. 4. BD-cellulose chromatography of [³H]trp-tRNA^{trp} extracted from *N. meningitidis* strain SD grown in MHB overnight and then transferred into fresh a, MHB for 3 h (—) and MHB-D for 3 h (.....); b, MHB-D for 6 h; c, MHB-D for 16 h. Ethanol gradient (-----). P1 and P2 = peaks 1 and 2.

Fig. 5. BD-cellulose chromatography of [^3H]trp-tRNA^{trp} extracted from *N. meningitidis* strain SD grown in MHB-TF for 24 h before (.....) and 6 h after (—) adding ferric di-citrate to the medium. Elution gradient (-----).

P2-dominant tRNA species pattern for MHB cultures to the P1-dominant tRNA species one for MHB-D cultures occurred slowly. Growth in MHB-D for up to 3 h showed no change, but at 6 h small but significant changes in the relative proportions of P1 and P2 were seen and the process was complete by 16 h (fig. 4).

When HTF was used to chelate the available iron in MHB, approximately equal amounts of tRNA in peaks P1 (46%) and P2 (48%) were obtained after overnight growth of the organisms (fig. 5). This profile was independent of the growth phase of the bacteria.

As it is known that in *E. coli* the early eluting tRNA species is a precursor of the late eluting one, it was important to see whether the P1 tRNA species was in any way related to the P2. Results showed that 6 h after transferring *N. meningitidis* grown in MHB-D to the iron-rich MHB (with added ferric di-citrate) no significant changes to the tRNA elution profile had occurred (fig. 6) despite the fact that the growth rate had recovered almost completely by that time (fig. 1). Even 24 h after transfer to iron-rich MHB, only partial recovery had been achieved, despite the fact that the bacteria had reached the stationary phase much earlier.

In contrast to that with the cells grown with desferrioxamine, within 6 h of adding sufficient iron to MHB-TF to oversaturate the iron-binding capacity of the HTF, the tRNA^{trp} elution profile had returned to

that with a minor P1 (22%) and major P2 (72%) peak (fig. 5).

Effects of other growth conditions

To determine whether any of the changes in tRNA^{trp} shown above could also be induced by other growth conditions, the *N. meningitidis* strain was grown overnight on the same medium solidified with agar (MHA) before the extraction of tRNA. The results showed that the chromatographic profile of trp-tRNA^{trp} from the organism grown on MHA was very similar to that produced when grown in MHB-D (see fig. 3). To see whether the alteration of the trp-tRNA profile from that given by cultures grown in MHB was due to lack of access to free iron or to the change in the growth environment from liquid to solid, ferric di-citrate (1%) was added to the MHA. This made no difference, and the major P1 and minor P2 tRNA pattern was again obtained. The incorporation of desferrioxamine into MHA (MHA-D) further suppressed the P2 tRNA species.

Discussion

The results show that changes occur in the tRNA^{trp} of *N. meningitidis* grown under iron-restricted conditions. However, it is important to note that the

Fig. 6. BD-cellulose chromatography of [^3H]trp-tRNA^{trp} extracted from *N. meningitidis* strain SD grown in MHB-D overnight (—) and then transferred into fresh MHB containing ferric di-citrate and incubated for 6 h (.....) and 24 h (-----). Elution gradient (---).

response to iron restriction by desferrioxamine and that produced by the presence of HTF in the medium was different; only the change produced by HTF seemed readily reversible and likely to reflect iron-related tRNA modification as seen in *E. coli*. Thus, the two systems are not identical and the use of desferrioxamine, although convenient, may not entirely mimic the more naturally produced iron-restricted environment imposed by the iron-binding protein. We suggest that desferrioxamine, an iron-chelator used widely experimentally,^{19, 23, 27} creates an artificial environment for the organisms where iron may be completely unavailable to the cells or where other trace elements, e.g., aluminium, copper, zinc and magnesium,²⁸ may also be limiting. Such stressful growth conditions could result in both general and specific effects on the formation of modified bases in tRNA, resulting in molecules that may be incapable of being converted to the normal species. Similar effects are seen when *E. coli* is cultured under stressful conditions, such as growth in the presence of chloramphenicol or in the absence of methionine, leucine or cysteine, which lead to the uncoupling of RNA and protein synthesis. The abnormal *E. coli* tRNA species that accumulate under such conditions, though chromatographically similar to those found in iron-restricted *E. coli*, are only converted to the normal species very slowly, or not at

all, on restoration of normal growth.²⁹⁻³³ Such abnormal tRNAs have a number of modified nucleosides missing as well as the methylthio-moiety of ms²i⁶A. However, it is also possible that two different and unrelated species of tRNA^{trp} exist in *N. meningitidis* and that one or other is preferentially expressed during these different growth conditions.

Our results show that *N. meningitidis* grown in a liquid medium is phenotypically different from that grown on solid medium. Although MHA has been reported to be relatively iron limited compared to MHB³⁴, in this case the tRNA changes seem not to be related to the lack of iron, although again it is not known if the chromatographically similar iso-accepting tRNA species seen are identical to those found during iron restriction.

A detailed analysis of the early and late eluting tRNA^{trp} species is now required to understand the molecular relationship between them and to see if iron restriction itself, as induced by HTF, results in the failure to methylthiolate an isopentenyladenosine in these tRNAs. Preliminary data (unpublished results) show that changes similar to those seen with tRNA^{trp} also occur in meningococcal phenylalanine and tyrosine tRNA under the same growth conditions. It remains to be seen whether any of these changes are connected with the adaptation of *N. meningitidis* to

growth in an iron-restricted environment, as appears to be the case in *E. coli*.

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