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Alterations in the tRNA's of *Escherichia coli* Recovered from Lethally Infected Animals

ELWYN GRIFFITHS,* JANICE HUMPHREYS, ANN LEACH, AND LINDA SCANLON
National Institute for Medical Research, Mill Hill, London NW7 1AA, England

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Escherichia coli grown in chemically defined iron-deficient media or in fluids containing the iron-binding proteins transferrin, lactoferrin, or ovotransferrin have well-characterized alterations in the chromatographic properties of tRNA's containing the modified nucleoside 2-methylthio-N^6-(Δ^2-isopentenyl)-adenosine. The present work shows that similar tRNA alterations occur in *E. coli* O111 recovered from the peritoneal cavities of lethally infected guinea pigs and rabbits. Adding iron to these in vivo-grown bacteria resulted in the rapid conversion of chromatographically abnormal tRNA's to the normal species. The work strongly suggests that host iron-binding proteins, present in mucosal and other secretions, can affect the metabolism of invading organisms. The idea that the tRNA alterations are connected with the adaptation of *E. coli* to growth under the iron restricted conditions imposed by iron-binding proteins in tissue fluids, and thus with bacterial pathogenicity, is therefore made particularly attractive.

Under conditions of iron deficiency, abnormal species of tRNA's appear in *Escherichia coli* which elute earlier on chromatography than the normal tRNA's (8, 18, 21). Iron is involved with the maturation of the hypermodified nucleoside 2-methylthio-N^6-(Δ^2-isopentenyl)-adenosine (ms^2i^6A), which is found next to the 3' end of the anticodon of these tRNA's. The chromatographically abnormal tRNA species from iron-deficient *E. coli* lack the methylthio moiety of this nucleoside (18). Similar chromatographic alterations have been shown to occur in the tRNA's of enteropathogenic *E. coli* growing rapidly in human milk, bovine colostrum, and in defined media containing the iron-binding protein transferrin or ovotransferrin (6). Adding iron to such bacteria resulted in the replacement of abnormal tRNA by the chromatographically normal species. It has been suggested that these tRNA alterations are connected with the adaptation of *E. coli* to the iron-restricted conditions imposed by the lactoferrin present in milk and colostrum, and by the iron-binding proteins added to the synthetic media (6). Since tRNA's are involved in cellular functions other than protein synthesis (12, 17) and since many body fluids, particularly mucosal secretions, contain transferrin and/or lactoferrin (1, 4, 14, 15), the very interesting possibility arises that changes in tRNA's induced by the presence of iron-binding proteins in tissue fluids may control some aspect of bacterial metabolism essential for pathogenicity (6).

In any work on infection it is essential that biochemical mechanisms studied in vitro are shown to be relevant; that is, that they also apply in vivo, in the animal body. If it is proposed that alterations in tRNA's play a role in the adaptation of *E. coli* to growth in vivo and also in their pathogenicity, then it must be shown that such changes occur in bacteria isolated directly from infected animals. In this communication we show that *E. coli* recovered from the peritoneal cavities of lethally infected animals do contain altered tRNA species.

MATERIALS AND METHODS

Bacteria. *E. coli* O111 K58 H2 was stored and grown as described previously (5). Cultures were harvested by centrifugation, and the organisms were resuspended in 0.15 M NaCl. The total cell count per milliliter was obtained by the use of a colorimeter and by estimating the value from a standard graph. Suitable dilutions for inoculations were made in 0.15 M NaCl. Viable counts were made on fresh blood agar plates.

Animals. Normal male or female Hartley strain guinea pigs (230 to 300 g) were obtained from the specific-pathogen-free colony at the National Institute for Medical Research.

Both normal and cannulated female New Zealand white rabbits were used. The technique used for fitting peritoneal cannula was that described by Bullen et al. (4).

Measurement of the iron-binding capacity of guinea pig peritoneal washings. Bacterial and tissue cells were removed by centrifugation from the pooled peritoneal washings from several guinea pigs, obtained as described below, and the supernatant was concentrated by ultrafiltration (4). Unsaturated iron-

binding capacities (UIBC) were determined as before (5), and the results are expressed in micromoles of Fe^{3+} per liter of the original peritoneal washings.

Intraperitoneal bacterial growth. (i) Guinea pigs. Groups of 10 to 14 guinea pigs were infected by intraperitoneal injections of bacteria suspended in 0.15 M NaCl (0.5 ml per animal). The animals were killed after suitable time intervals with CO_2 gas. A 30-ml amount of 0.15 M NaCl was injected into the peritoneum of each animal and, after kneading the abdomen, the fluid was carefully removed with a pipette through an incision in the abdominal wall. The total bacterial viable count per ml of exudate was measured after a sample of the peritoneal washings had been homogenized for 7 min in an ice bath.

(ii) Rabbits. Individual rabbits were infected under anaesthesia (halothane/nitrous oxide) by introducing bacteria suspended in 10 ml of 0.15 M NaCl via the cannula by using a perforated tube. This was followed with 5 ml of saline to rinse the tube. The rabbits were then allowed to recover from the anaesthetic. Samples of peritoneal fluid for estimating the viable count were obtained, again under anaesthesia, by introducing 30 ml of heparinized saline (0.08 mg of heparin per ml) via the cannula, kneading the abdomen gently for 1 min and removing 10 ml of exudate with a perforated tube. The total viable count per milliliter of exudate sample was obtained after the recovered fluid was homogenized as described by Bullen et al. (4). When isolating bacteria for the preparation of tRNA's, larger volumes of heparinized saline (80 ml) were introduced into the peritoneum, and, after kneading the abdomen, as much fluid as possible was removed. The rabbits were then allowed to recover from the anaesthetic, but subsequently died from the infection within 24 h. Some rabbit experiments were carried out with normal, noncannulated animals. Individual rabbits were infected by an intraperitoneal injection of bacteria suspended in saline. The animals were killed after a suitable time interval with CO_2 gas, and the bacteria were collected by washing the peritoneum with heparinized saline (100 ml) in the same way as described above for guinea pigs.

The rabbit and guinea pig peritoneal washings were centrifuged at 100 × *g* for 10 min to sediment tissue cells. Viable counts of the supernatant fluid gave a measure of the extracellular bacteria.

Examination of bacterial aminoacyl tRNA's. Bacteria in the peritoneal washings from individual rabbits or in the pooled peritoneal washings from several guinea pigs were collected by centrifugation at 0 to 5°C after first removing tissue cells. The bacteria were washed in 0.15 M NaCl, 0.01 M $NaHCO_3$, and 0.1% (wt/vol) glucose (pH 7.6). Traces of erythrocytes were sometimes present at this stage, but these were easily removed by centrifuging the bacterial suspension at top speed in a bench centrifuge for 0.5 to 1 min. Washed *E. coli* O111 were resuspended in more 0.15 M NaCl, 0.01 M $NaHCO_3$, and 0.1% (wt/vol) glucose (pH 7.6), and their tRNA was aminoacylated with ^{14}C- or ^{3}H-labeled phenylalanine or tryptophan, isolated and fractionated by chromatography on benzoylated diethylaminoethyl-cellulose (BD-cellulose; Bio-Rad Laboratories) as described by Griffiths and Humphreys (6). L-[*G*-^{3}H]tryptophan (4.9 Ci/mmol), DL-[methylene-^{14}C]tryptophan (53 Ci/mol), L-[*G*-^{3}H]-phenylalanine (1.0 Ci/mmol), and L-[*U*-^{14}C]phenylalanine (531 Ci/mol) were obtained from the Radiochemical Centre (Amersham, United Kingdom).

E. coli O111 from which normal aminoacyl-tRNA's were to be isolated was grown for 3 h at 37°C in papain digest broth or Trypticase soy broth (Baltimore Biological Laboratory; Becton, Dickinson & Co.). These broth-grown cells were harvested by centrifugation, washed in 0.15 M NaCl, 0.01 M $NaHCO_3$, and 0.1% (wt/vol) glucose (pH 7.6), and their tRNA's were aminoacylated, isolated, and chromatographed on BD-cellulose as described previously (6). The preparation of aminoacyl-tRNA's from *E. coli* O111 grown in Trypticase soy broth containing ovotransferrin (Sigma Chemical Co., type I) (1 mg/ml) has also been described before (6). Incubation of in vivo-grown *E. coli* O111 in Trypticase soy broth with or without ovotransferrin (1 mg/ml) was carried out in 10-ml volumes under controlled conditions (6). After incubation the bacteria were washed and their tRNA's were aminoacylated as described above.

In dual label experiments, the ^{3}H-labeled and ^{14}C-labeled aminoacyl-tRNA's were mixed together before being co-chromatographed on the BD-cellulose.

Elution diagrams 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. The relative amounts of normal and abnormal tRNA's were calculated from the total radioactivity present in each peak and expressed as a percentage of the total radioactivity recovered.

RESULTS

Guinea pig experiments. Previous work has shown that the intraperitoneal injection of about 10^8 *E. coli* O111 into normal young adult guinea pigs invariably killed the animals within 48 h (3). A similar lethal dose was used in the present work. At specific times after infection, the guinea pigs were killed and the bacteria were recovered from their peritoneal cavities. It was clear from the numbers of bacteria obtained in this way that the organisms were multiplying rapidly in vivo. Most of the viable bacteria (80 to 90%) were extracellular during the first 8 h of infection. Figure 1 shows the number of extracellular *E. coli* O111 found in the guinea pig peritoneum at different times after infection. From the viable counts, an apparent generation time of about 60 min can be calculated. Only the extracellular bacteria were used for studying the tRNA's. Most animals were visibly ill, moribund, or dead 16 h postinfection. The injection of 10^8 heat-killed *E. coli* O111 had no apparent effect on the guinea pigs, and all animals were alive when examined approximately 24 h later. Results from several experiments showed that the UIBC of peritoneal washings from infected animals (14 to 15 h after infection) was 4 to 5 μmol of Fe^{3+} per liter and that from normal animals was 0.2 to 0.3

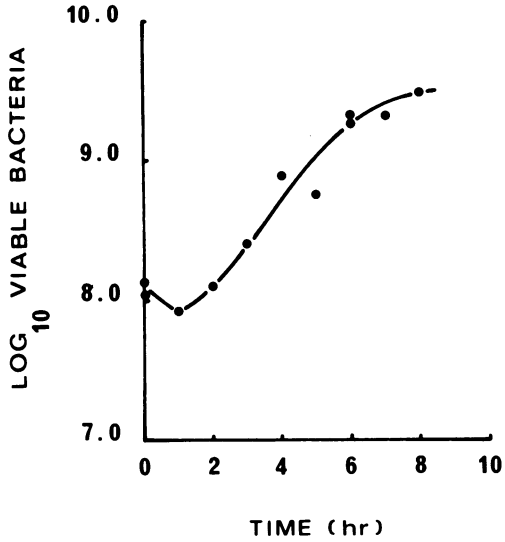

FIG. 1. *Growth of E. coli O111 in the guinea pig peritoneum. Each point represents the mean of the extracellular viable bacterial count from two animals.*

μmol of Fe^{3+} per liter.

The elution profiles of tryptophanyl-tRNA (Trp-tRNA) extracted from *E. coli* O111 isolated from the peritoneal cavities of sick or moribund guinea pigs (14 to 15 h after infection), from *E. coli* O111 grown in broth, and from *E. coli* O111 grown in Trypticase soy broth containing ovotransferrin (1 mg/ml) are shown in Fig. 2. On BD-cellulose chromatography, Trp-tRNA from normal, broth-grown, iron-replete *E. coli* O111 eluted as a single peak (Fig. 2A). Trp-tRNA from the in vivo-grown organisms, however, showed a new distinct major peak eluting well ahead of the normal position, together with a small peak of the normal species. A dual label experiment, involving co-chromatography of ^{14}C- and ^{3}H-labeled aminoacyl-tRNA's, showed that the chromatographically abnormal Trp-tRNA from the in vivo-grown *E. coli* O111 coeluted with the abnormal Trp-tRNA extracted from the bacteria grown in Trypticase soy broth containing ovotransferrin (1 mg/ml) (Fig. 2B). In fact, the two elution profiles were nearly identical, with over 90% of the Trp-tRNA eluting as the altered species in both cases. Similar results were obtained with phenylalanyl-tRNA (Phe-tRNA). Again, the abnormal Phe-tRNA obtained from the in vivo-grown bacteria coeluted with the altered Phe-tRNA found in *E. coli* O111 grown in Trypticase soy broth containing ovotransferrin (1 mg/ml). Most (80 to 90%) of Phe-tRNA extracted from *E. coli* O111 isolated from the peritoneal cavities of sick or moribund guinea pigs was abnormal. *E. coli* O111 which had been grown intraperitoneally for only 5 h contained about 70% of the altered tRNA species.

When the in vivo-grown bacteria were resuspended in Trypticase soy broth containing added Fe^{3+} (0.05 mM), the abnormal tRNA's reverted rapidly to the chromatographically normal species. Figure 3 shows the conversion of abnormal Phe-tRNA to normal on resuspension of in vivo-grown *E. coli* O111 in Trypticase soy broth containing Fe^{3+}; after a 50-min incubation, 82% of Phe-tRNA eluted in the chromatographically normal position. That this conversion was indeed due to the availability of Fe^{3+} was shown by the fact that conversion did not occur when in vivo-grown *E. coli* O111 was incubated for 60 min in Trypticase soy broth containing ovotransferrin (1 mg/ml), but did occur when sufficient iron was then added to this medium to oversaturate the iron-binding capacity of the ovotransferrin present. Iron was added as ferric dicitrate (6).

Rabbit experiments. Results similar to those obtained with infected guinea pigs were obtained with lethally infected rabbits. A lethal dose of *E. coli* O111, ca. 10^{10} bacterial cells, was introduced into the rabbit peritoneum via a cannula. Sampling the peritoneal cavity subsequent to infection showed that *E. coli* O111 grew in this location, and the rabbits died from the infection within 24 h. The total number of bacteria per rabbit peritoneum increased from about 3×10^{10} organisms at 2 h postinfection to over 10^{11} organisms at 5 h. Approximately 10^{11} extracellular bacteria per rabbit were recovered for examination of the tRNA's. Results showed that after 5 h of growth in the peritoneum of cannulated rabbits, *E. coli* O111 contained about 60% of abnormal Trp-tRNA and Phe-tRNA. *E. coli* O111 isolated after growth in the peritoneal cavity of normal, noncannulated rabbits for 5 h contained 72% abnormal Trp-tRNA and 60% abnormal Phe-tRNA. These were results from individual rabbits, and they resemble those found with *E. coli* O111 grown in guinea pig peritoneum for 5 h. The introduction of 10^{10} heat-killed *E. coli* O111 into the peritoneal cavities of either cannulated or normal rabbits had no apparent effect on the animals, and all were alive 48 h later.

DISCUSSION

E. coli grown in chemically defined iron-deficient media, in human milk, in bovine colostrum, or in media containing the iron-binding protein transferrin or ovotransferrin have well-characterized alterations in the chromatographic prop-

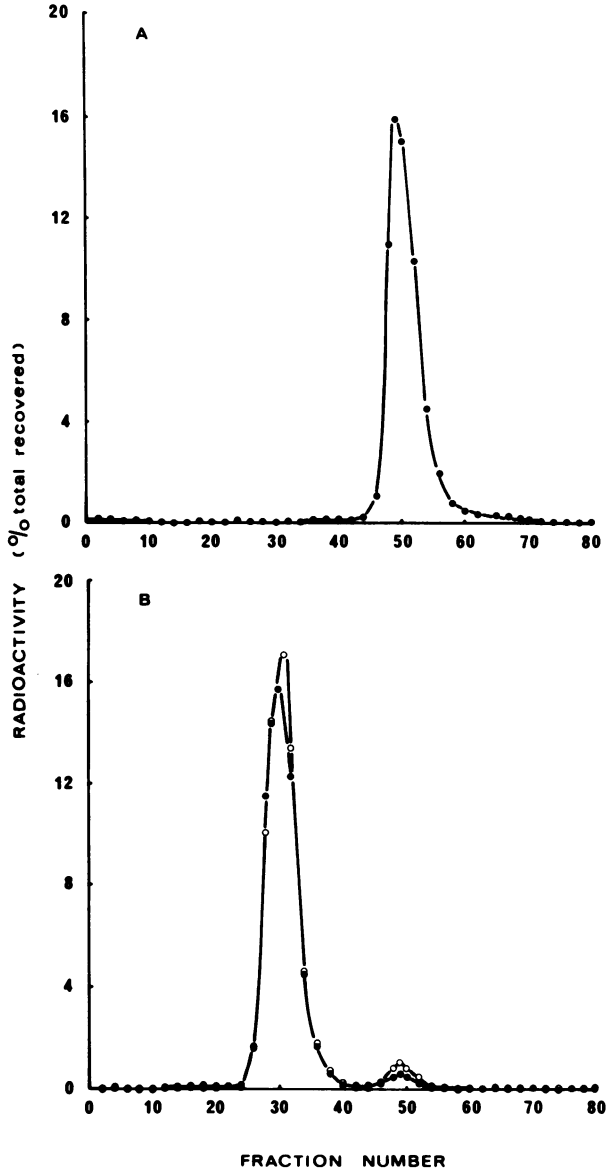

FIG. 2. *BD-cellulose chromatography of Trp-tRNA. (A) [^{3}H]Trp-tRNA extracted from iron-replete broth-grown E. coli O111. (B) Co-chromatography of* (○) *[^{14}C]Trp-tRNA isolated from E. coli O111 grown in the guinea-pig peritoneum and* (●) *[^{3}H]Trp-tRNA from E. coli O111 grown in Trypticase soy broth containing ovotransferrin (1 mg/ml). (A) was chromatographed separately on the same column.*

erties of tRNA's containing the hypermodified nucleoside ms^{2}i^{6}A (6, 8, 18, 21). Under iron-restricted conditions, abnormal species of the tRNA's appear which elute earlier on chromatography than do the normal species. The tRNA's involved are those for phenylalanine, tyrosine, tryptophan, serine, cysteine, and leucine, all of which recognize codons with a 5'-terminal uridine (UNN'). The present work shows that two of these tRNA's are similarly altered in *E. coli* O111 recovered from the peritoneal cavities of lethally infected guinea pigs and rabbits. The other tRNA's have not yet been examined, but it is likely that they would also behave in the same way. Iron specifically affects the synthesis of the methylthio group on the isopentenyladenosine (i^{6}A) in tRNA (18). On adding iron to the in vivo-grown *E. coli* O111, the chromatographically abnormal tRNA's reverted to the normal species at a rate similar to

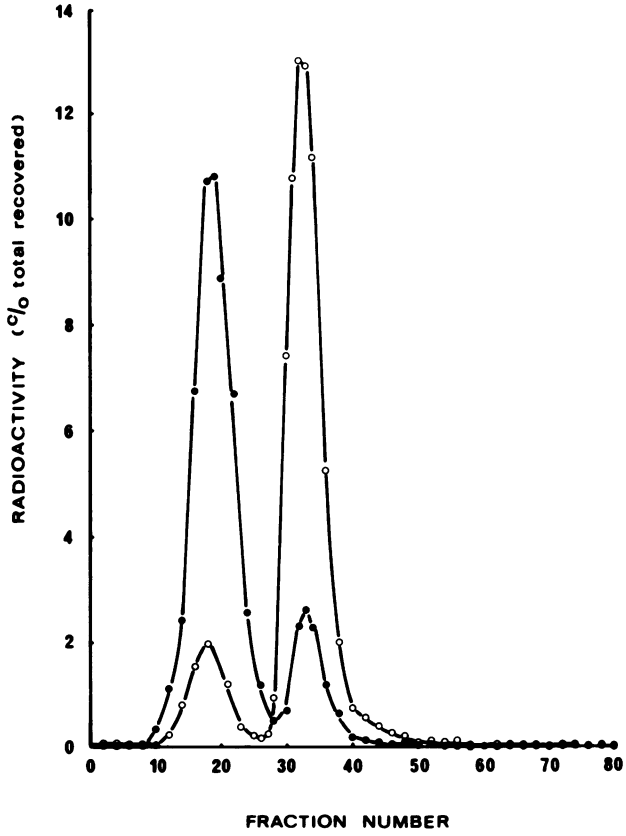

FIG. 3. *Replacement of abnormal [^{3}H]Phe-tRNA by the normal species on incubating guinea-pig grown E. coli O111 in Trypticase soy broth containing added Fe^{3+} (0.05 mM). Symbols: ●, before incubation (time zero); ○, after 50 min of incubation.*

that found on adding iron to *E. coli* O111 grown in media containing iron-binding proteins (6). This strongly suggests that the altered chromatographic behavior of Trp- and Phe-tRNA from the in vivo-grown *E. coli* O111 results from a failure to methylthiolate isopentenyladenosine (i^6A) after a disturbance in bacterial iron metabolism. Abnormal species of tRNA's which appear in *E. coli* after inhibition of protein synthesis either by chloramphenicol or by leucine starvation of relaxed control *E. coli*, although chromatographically similar to those found in iron-restricted bacteria, are converted to the normal species only very slowly, over a 3 to 4 h period, or not at all, on restoration of growth (8, 9, 11, 13). Such abnormal tRNA's have other modified nucleosides missing as well as the methylthio moiety of ms^2i^6A (7, 10, 11). Definitive evidence for the suggested difference between the chromatographically normal and abnormal tRNA's described in this paper is currently being pursued.

These results are, of course, those which would be expected if the iron-binding proteins present in the secretions of the peritoneal cavity exert an effect on bacterial metabolism. Rabbit peritoneal washings have a measurable unsaturated iron-binding capacity, although it is not known whether this is due to transferrin, lactoferrin, or to a mixture of both (4). Similarly, guinea pig peritoneal washings have an unsaturated iron-binding capacity, the value of which increases significantly on infection. This could be due to serum transferrin, increasing amounts of which may move into the peritoneal cavity through increased permeability after infection (19), and/or due to the release of lactoferrin from neutrophilic polymorphonuclear leukocytes (16, 20). The release of lactoferrin from polymorphs is part of the proposed mechanism for producing the hyposideremia of infection (20).

Our work shows, therefore, that *E. coli* O111 growing in vivo and producing a lethal infection contains tRNA's with properties similar to those found in the bacteria growing in the presence of

iron-binding proteins and which are considerably different from those found in normal broth-grown organisms. This clearly suggests that host iron-binding proteins, present in mucosal secretions and other body fluids, can indeed affect the metabolism of invading organisms. The data do not by themselves show that the appearance of altered tRNA species is necessary for the infection to occur in normal animals. However, given the numerous and varied roles of tRNA's in bacterial cells (2, 12, 17), the idea that the tRNA alterations are connected with the adaptation of *E. coli* to growth on or in host tissues, and consequently with their pathogenicity (6), is very attractive and can, we feel, now be investigated with confidence.

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