Formylmethionyl-leucylphenylalanine and the SOS operon in *Escherichia coli*: a model of host–bacterial interactions

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To determine the biological significance of the existence of highly specific receptors for the bacterial chemotactic peptide formylmethionyl-leucylphenylalanine (fMet-Leu-Phe) on neutrophil leucocytes, we investigated the role of this peptide in bacterial metabolism. The UmuD protein of the *Escherichia coli* SOS operon was identified as having an N-terminal fMet-Leu-Phe sequence and a recombinant *E. coli* with the *umuD* gene on plasmid pSB13 was shown to be an over-producer of both UmuD and fMet-Leu-Phe. Activation of SOS genes in conventional wild-type *E. coli* (K12) by u.v. light or hydrogen

peroxide increased fMet-Leu-Phe production up to 4-fold. A *RecA*[−] strain, incapable of SOS activation, was a low basal producer of fMet-Leu-Phe and showed no increased production with u.v. light or oxidant stress. We propose that host phagocytes respond to fMet-Leu-Phe and closely related peptides because they are generated by bacteria under oxidant stress. Increased fMet-Leu-Phe production may signal to the host a change in the organism's biological status from commensal to pathogen because of the invasion into tissues exposing bacteria to high pO₂ levels and oxidant stress.

INTRODUCTION

The existence of highly specific receptors for formylmethionyl-leucylphenylalanine (fMet-Leu-Phe) and closely related peptides on granulocytes, macrophages, endothelial cells and activated B-lymphocytes suggests an important role for this peptide and its receptor in host detection of bacterial metabolism [1–6]. However, the identity of the fMet-Leu-Phe precursor protein and thus its role in bacterial metabolism have remained a mystery, and therefore the biological significance of the fMet-Leu-Phe–host interaction has remained obscure. Indeed, since several species do not possess formyl-peptide receptors it has been argued that they may subserve a function other than the detection of bacterial metabolites, and that an endogenous host ligand could be the primary agonist [7].

In spite of uncertainty regarding the significance of the formyl-peptide receptor structure–activity studies strongly suggest that fMet-Leu-Phe is the primary agonist, eliciting chemotaxis with an EC₅₀ of 7×10^{-11} M and lysosomal enzyme secretion with an EC₅₀ of 2.5×10^{-10} M (rabbit neutrophils) [7,8]. The formyl group is essential for biological activity, as is the methionine which is postulated to fit into a hydrophobic pocket such that the sulphur interacts with a discrete region of positive charge. The leucyl side-chain hydrophobic pocket is relatively loose around the β carbon and can tolerate substitutions of norvaline, valine or isoleucine. The hydrophobic pocket accommodating the aromatic ring of phenylalanine seems to be highly specific; there is a dramatic reduction of biological potency by the introduction of a *p*-hydroxyl group to form tyrosine. The presence of a fourth hydrophobic pocket is supported by the increase in potency of fMet-Leu-Phe-(Ile/Phe) and by the reduced potency of a carboxyl Lys.

In pursuit of a candidate precursor for fMet-Leu-Phe we searched the Protein Identification Resource database (PIR) for any N-terminal Met-Leu-Phe sequence (assuming fMet initiation in bacterial proteins). A single candidate, the UmuD protein of the *E. coli* SOS operon, was identified. The SOS operon provides for DNA repair in bacteria [9–11]. Agents such as u.v. light [11],

hydrogen peroxide [12] or oxygen free radicals [13] damage double-stranded DNA and expose segments of single-stranded DNA triggering expression of at least 17 SOS genes. Some of the proteins synthesized in response to DNA damage participate in excision repair, whereas others such as UmuC and UmuD reduce the fidelity of DNA replication, allowing bypass of the site of DNA damage with elevated levels of mutations.

In this paper we demonstrate that UmuD is a precursor of fMet-Leu-Phe and that SOS activation in *E. coli* leads to elevated fMet-Leu-Phe production. These findings enabled us to develop a hypothesis concerning the biological significance of the formyl-peptide receptor in host bacterial interactions.

MATERIALS AND METHODS

Reagents

Ampicillin and tetracycline were obtained from Sigma. Acrylamide, Tris, glycine, Tween 20, gelatin (EIA grade) and nitrocellulose were from Bio-Rad. [¹²⁵I]iodine and [⁵⁷Co]cyanocobalamin (vitamin B₁₂) were from Amersham.

Anti-fMet-Leu-Phe antiserum (polyclonal) was obtained following immunization of rabbits with fMet-Leu-Phe–BSA conjugate. The antibodies were purified by affinity purification on fMet-Leu-Phe–Sephacrose. The specificity of the antibodies has been reported previously [14]. Biotinylated anti-[rabbit IgF(ab')₂] antibody and streptavidin–peroxidase complex from Amersham were used for detection of the primary antibody on Western blots. Carboxypeptidase Y (from yeast) used for C-terminal peptide micro-sequencing was from Boehringer–Mannheim.

Bacterial strains

Strain MC1000 (pRK248, pSB13) was a kind gift from Dr. N. A. Petit, Dr. Echols Laboratory, Department of Molecular and Cell Biology, University of California, Berkeley, CA, U.S.A. [15]. This strain was chosen as it over-expresses UmuD. The *umuD* and *umuC* genes are contained within plasmid pSB13 and are controlled by the temperature-regulated P_L promoter rather

Abbreviation used: fMet-Leu-Phe, formylmethionyl-leucylphenylalanine.

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than via the SOS promoter. Standard wild-type *E. coli* K12 and the *E. coli* hospital isolates were obtained from the Microbiological Services Department of the Dunedin Public Hospital, Dunedin, New Zealand. *E. coli* strain DH5 α [*RecA*⁻, *F*⁻, *end A1*, *gyr A96*, *thi-1*, *hsd R17* (*r_k*⁻*m_k*⁺) *sup E44*, *rel A1*] was originally obtained from Clontech Laboratories Inc., CA, U.S.A. and was chosen because it lacks the enzyme *RecA* and therefore cannot cleave LexA, the repressor of the SOS operon.

Bacterial culture

Bacteria were cultivated in RPMI-1640 cell-culture medium (Sigma) to mid-logarithmic phase ($1-2 \times 10^8$ cells/ml). RPMI synthetic media promoted good bacterial growth and were free of peptides and proteins which could have interfered with r.i.a. of highly concentrated culture supernatants. The cells were harvested by centrifugation (10000 *g* for 10 min at 4 °C) for identification of the fMet-Leu-Phe precursor protein and the supernatants removed (90 % vol. taken to avoid disturbing pellet) for isolation and assay of extracellular formyl peptides.

Immunodetection of the fMet-Leu-Phe precursor protein

Bacterial samples were prepared for electrophoresis by boiling (3 min) in sample buffer which contained 3 % (w/v) SDS, 8 M urea, 70 mM dithiothreitol, 1 % (w/v) Bromophenol Blue and 25 mM Tris/HCl (pH 6.7). The cells were subjected to three freeze/thaw (-70 °C/100 °C) cycles and finally reboiled (3 min) before electrophoresis. SDS/PAGE was performed using the procedure of Laemmli [16] and the Bio-Rad mini-gel apparatus. The separating gel was 15 % (w/v) and the stacking gel was 3.5 % (w/v) acrylamide. Western blotting on to nitrocellulose was performed as described in the Bio-Rad electrophoresis-transfer-cell instruction manual using 25 mM Tris/192 mM glycine, 20 % (v/v) methanol, pH 8.3. Gelatin (0.5 % w/v) in Tris-buffered saline (TBS) was used to block the nitrocellulose and the gels were probed using affinity-purified polyclonal anti-fMet-Leu-Phe antibodies. Bound antibody was detected using biotinylated anti-(rabbit antibody) antisera followed by strept-avidin complex and colour development using 4-chloro-1-naphthol as substrate.

H.p.l.c. fractionation of bacterial supernatants

Cell-free bacterial culture supernatants (volumes 10–50 ml) were diluted 1:1 with a mixture containing acetonitrile/water/trifluoroacetic acid (10:90:0.1, by vol.) (buffer A), loaded on to Varian Analytichem Bond elut (C₁₈, 6 bed volume) cartridges and washed further with 10 ml of the same solution. Elution of the peptides of interest was by washing with acetonitrile/water/trifluoroacetic acid (60:40:0.1, by vol.) (buffer B). This material was freeze-dried and resuspended in PBS (10 mM sodium phosphate/150 mM sodium chloride, pH 7.5) for r.i.a. and bioassay (see below). Reverse-phase fractionation of the material was performed using a Hypersil C₁₈ column and a gradient of 10–60 % acetonitrile (v/v), 0.1 % (v/v) trifluoroacetic acid, with a flow rate of 1 ml/min as described previously [17]. The fractions were freeze-dried and redissolved in PBS for r.i.a. and bioassay.

Radioimmunoassay and bioassay of fMet-Leu-Phe

R.i.a. and bioassay (secretion of vitamin B₁₂-binding protein by human neutrophils after challenge with h.p.l.c. fractions) of fMet-Leu-Phe have been described previously [14,18].

C-terminal sequencing of carboxypeptidase Y

Verification of the structure of immunoreactive peptides from bacterial culture supernatants was by C-terminal microsequencing, performed essentially as described previously [19]. The immunoreactive fractions, after reverse-phase chromatography, were resuspended in 400 μ l of 100 mM sodium phosphate, pH 6.2. Carboxypeptidase Y concentration was optimized with synthetic fMet-Leu-Phe (500 pmol) so that stoichiometric release of leucine and phenylalanine occurred within 5–15 min. The procedure for sequencing of the immunoreactive factors was as follows. Sample (50 μ l) was equilibrated to 25 °C and carboxypeptidase Y (20 μ l) was added. At various time intervals (0.5, 6, 60 min) 50 μ l of borate buffer was added and the samples boiled for 2 min to inactivate the enzyme. The amino acids released were analysed as their fluorescent orthophthaldialdehyde derivatives (excitation wavelength 325 nm, emission wavelength 425 nm) after reverse-phase chromatography [20]. A zero-time control containing enzyme and buffer was boiled before addition of substrate. Autodigestion of the enzyme was also monitored by using an enzyme/buffer incubation for 60 min.

Oxidative-stress experiments

E. coli cultures grown overnight in RPMI 1640 medium were diluted out in fresh medium and grown to a *D*₅₂₅ value of 0.3. The cells were then harvested by centrifugation at 8000 *g* for 7 min and resuspended in an equal volume of fresh medium pre-equilibrated to 37 °C. Hydrogen peroxide was added, the cells were then incubated with shaking for 90 min at 37 °C, and then harvested by centrifugation. The culture supernatant was then processed for analysis of fMet-Leu-Phe. Experiments involving continuous infusion were performed in a similar manner, except hydrogen peroxide was delivered continuously using an LKB Microperpex 2132 peristaltic pump at 3 ml/h and a hydrogen peroxide concentration of 1 mM. After 60 min the cells were harvested and the culture supernatants processed. The culture densities were determined using turbidities at 525 nm.

U.v. irradiation

U.v. activation of the SOS operon was performed by growing freshly diluted cells to an attenuance of 0.2 and transferring 50 ml aliquots of this culture into a 23 cm $\times$ 16 cm plastic dish supported within a 37 °C waterbath. A pre-u.v.-treatment control sample was processed immediately while cells were irradiated. The u.v. source was a Philips 30 W TUV G30T8 u.v. lamp mounted 1.2 m above the cell suspension. Exposure was generally for 10–60 s; a 10 s exposure in this system delivered approx. 0.5 J/m². The cells were then diluted 1:1 in fresh medium which had not been exposed to u.v. light and allowed to grow until the individual cultures (different doses of u.v.) reached an attenuance value of 0.36. The cells were then harvested and the culture supernatants processed for measurement of fMet-Leu-Phe concentrations (see above).

RESULTS

UmuD protein as a precursor of fMet-Leu-Phe

A search of the Protein Identification Resource database (PIR) [21] had revealed that the *E. coli* UmuD protein was a candidate protein precursor of the leucocyte chemoattractant fMet-Leu-

Figure 1 N-terminal sequence and RecA-cleavage site of the *E. coli* UmuD protein

The amino-acid sequence of *E. coli* UmuD protein has been predicted from the DNA sequence data [10].

Figure 2 Western blot and immunodetection of UmuD

Total cell lysates from *E. coli* strain MC1000 (pSB13) grown at 42 °C and 30 °C were fractionated by SDS/PAGE, blotted on to nitrocellulose and probed with anti-(fMet-Leu-Phe) antiserum. Lane 1, 42 °C immunodetection; lane 2, 30 °C immunodetection; lane 3, Coomassie Blue stain, 42 °C; lane 4, Coomassie Blue stain, 30 °C; lane 5, molecular-mass markers.

Phe. The amino-acid sequence of the *E. coli* UmuD protein had been predicted from the DNA sequence. UmuD is activated by RecA cleavage at position 24/25 (Figure 1), excising a 3 kDa N-terminal fragment and releasing a 12 kDa UmuD'-active protein [11,12,22].

Using *E. coli* MC1000 pRK248 pSB13, the 15 kDa UmuD protein was detected on Western blots with affinity-purified anti-fMet-Leu-Phe polyclonal antibodies when the organisms were grown at the permissive temperature, but not when grown at non-permissive temperatures (Figure 2). Using a r.i.a. for fMet-

Table 1 Endogenous secretion of fMet-Leu-Phe immunoreactivity

Different strains of *E. coli* were assessed for fMet-Leu-Phe secretion during mid-logarithmic-phase growth. The strains and the procedure are described in detail in the Materials and methods section. Abbreviations: *n*, number of determinations; Hos, hospital clinical isolate.

<i>E. coli</i> strain	fMet-Leu-Phe equivalents (pmol/h per 10 ⁹ cells)	<i>n</i>	Temperature (°C)
K12	2.47 (S.D. 0.29)	8	37
Hos1	5.4	1	37
Hos2	6.8	1	37
Hos3	2.6	1	37
MC1000 (pSB13)	226.3 (S.D. 14.13)	3	42
MC1000 (pSB13)	43.2 (S.D. 3.72)	3	40
MC1000 (pSB13)	1.2	1	30
DH5a	0.44 (S.D. 0.23)	3	37

Figure 3 H.p.l.c. (C₁₈) chromatography of immunoreactive and bioactive peptides from *E. coli* MC1000 (pSB13)

Immunoreactive material secreted by strain MC1000 (pSB13) at 42 °C was fractionated by reverse-phase (C₁₈) chromatography. The fractions were assayed for fMet-Leu-Phe immunoreactivity (a) and bioactivity (b) (stimulation of neutrophil release of vitamin B₁₂ binding protein). Synthetic fMet-Leu-Phe elutes in fractions 25/26. Only the window of interest is shown; fractions below no. 22 and above no. 35 were assayed and showed no detectable material at the dilution required for optimal measurement of the two major peaks in both assays

Leu-Phe we demonstrated a parallel increase in fMet-Leu-Phe immunoreactivity in culture supernatants of the recombinant strain grown at the permissive temperature, but not at the non-permissive temperature. A comparison of fMet-Leu-Phe secretion rates among different *E. coli* strains is shown in Table 1.

Reverse-phase chromatography (C₁₈) of the immunoreactive factors secreted by strain MC1000 (pRK248, pSB13) at 42 °C identified two separate components, one which co-migrated with synthetic fMet-Leu-Phe and another more hydrophobic species (Figure 3a). Carboxypeptidase Y microsequencing verified the structures fMet-Leu-Phe and fMet-Leu-Phe-Ile respectively, corresponding to the first three and four amino-acid residues of the UmuD protein (Figure 4). Bioassay of fractions containing these peptides showed that both peptides were potent activators of human polymorphonuclear leucocytes (Figure 3b).

Effect of SOS activation on fMet-Leu-Phe production in conventional *E. coli*

To test whether SOS activation in conventional *E. coli* leads to elevated secretion of fMet-Leu-Phe, we subjected the K12 strain to hydrogen peroxide stress. The optimum concentration for hydrogen peroxide treatment of *E. coli* K12 was 10 μM, resulting in a 2.7-fold increase in secreted immunoreactivity (Figure 5). Under our experimental conditions mid-logarithmic phase *E. coli* consumed 10 μM hydrogen peroxide within a 10 min period. When these cells were subjected to a continuous infusion of 1 μM hydrogen peroxide/min over a 1 h period the secretion of

Figure 4 Carboxypeptidase Y microsequencing of immunoreactive/bioactive fractions 25/26 and 31

Immunoreactive/bioactive fractions from the C_{18} reverse-phase chromatography of *E. coli* MC1000 (pSB13) culture filtrate (42 °C induction) were subjected to carboxypeptidase Y microsequencing (see details in the Materials and methods section). The left-hand column shows the sequential digests and amino-acid analysis of fractions 25/26. The traces from top to bottom are the amino-acid analyses after 0 min digest, 0.5 min digest, 6 min digest and 60 min digest. The right-hand column is the sequential digest of fraction 31. The traces from top to bottom are 0 min digest, 0.5 min digest ($\frac{1}{4}$ strength enzyme), 0.5 min digest, 6 min digest, 60 min digest.

extracellular fMet-Leu-Phe was 3.5 times greater than control levels. Chloramphenicol treatment inhibited the hydrogen peroxide effect. Reverse-phase fractionation and r.i.a. showed that immunoreactivity was associated with elevated levels of fMet-Leu-Phe (fractions 24/25) but not of fMet-Leu-Phe-Ile (fractions 30/31) [Figures 6(a) and 6(b)]. The structure of the antigenic material eluting in fractions 16/17 has not been identified. A *RecA*⁻ strain of *E. coli* (DH5a) was a very low producer of fMet-Leu-Phe immunoreactivity under basal conditions (Table 1), and did not show significantly increased fMet-Leu-Phe secretion in response to hydrogen peroxide treatment [Figures 6(c) and 6(d)].

To further examine whether SOS activation led to elevated fMet-Leu-Phe secretion we subjected *E. coli* K12 to sublethal doses of u.v. irradiation (Figure 7). A maximum 4.3-fold elevation of fMet-Leu-Phe secretion was observed after a 10 s exposure to u.v. light (0.5 J/m²), which resulted in a 29% reduction in growth rate.

DISCUSSION

Activation of the SOS operon in *E. coli* can be triggered by the DNA-damaging effects of u.v. irradiation, by addition of exogenous hydrogen peroxide, or by drugs such as mitomycin C, bleomycin and nalidixic acid [23]. The proteins RecA, UmuD and UmuC reduce the fidelity of DNA replication, enabling continued base incorporation through the sites of DNA damage [23,24]. RecA plays a key role as a sensor of single-stranded DNA and as a specific protease, inactivating LexA, the repressor protein of the SOS operon. In addition, RecA activates the 15 kDa UmuD polypeptide by excision of the 3 kDa N-terminal fragment with an fMet-Leu-Phe initial sequence, yielding the 12 kDa UmuD' active protein [10,11,22].

To show that UmuD was a precursor for fMet-Leu-Phe we used a recombinant MC1000 strain containing the *umuD* gene with a heat-sensitive λ repressor on plasmid pSB13. At the permissive temperature, synthesis of UmuD protein was demonstrated and at the same time fMet-Leu-Phe and fMet-Leu-Phe-Ile appeared in the culture supernatant. In non-recombinant strains fMet-Leu-Phe is the only predominant extracellular product, suggesting that in the recombinant strain proteolytic machinery is overloaded, resulting in incomplete processing. We therefore propose that in *E. coli* UmuD protein is a source of the chemoattractant peptide fMet-Leu-Phe.

In the absence of DNA-damaging stimuli, basal secretion of fMet-Leu-Phe by *E. coli* may arise from low-level transcription of the *umuD* gene. UmuD is synthesized in non-SOS-induced *E. coli* cells which are not expressing other SOS-dependent genes [25] and it has been estimated that there are approx. 180 molecules of UmuD per cell. UmuD turnover in the non-stressed cell may occur via basal RecA activation or via totally different routes not involving RecA activation nor formation of UmuD' but still ultimately resulting in fMet-Leu-Phe production. This suggestion is consistent with the observation that the unstressed recombinant *E. coli* strain MC1000 pSB13, at the permissive temperature, is an over-producer of UmuD but not UmuD' [15], at the same time secreting high levels of fMet-Leu-Phe and fMet-Leu-Phe-Ile (see Figure 2). Even when the temperature was reduced from 42 °C to 40 °C this strain secreted high levels of fMet-Leu-Phe and fMet-Leu-Phe-Ile, suggesting activity of a UmuD-degradative pathway which bypasses UmuD' production and which was not simply a result of activation of heat-shock proteases.

Figure 5 The effects of hydrogen peroxide on secretion of fMet-Leu-Phe

Mid-logarithmic-phase *E. coli* strain K12 was exposed to a single dose of hydrogen peroxide and then harvested after 90 min. The fMet-Leu-Phe (fMLP) equivalents in the culture medium were determined as described in the Materials and methods section. CAP indicates addition of chloramphenicol (170 μ g/ml). C indicates a continuous infusion of hydrogen peroxide.

Figure 6 Reverse-phase chromatography and r.l.a. of peptides secreted by *E. coli* after hydrogen peroxide stress

E. coli strains K12 and DH5a were exposed to hydrogen peroxide stress and harvested after 90 min. The peptides were recovered from the culture medium, fractionated by reverse-phase chromatography and assayed for fMet-Leu-Phe (fMLP) immunoreactivity. (a), K12 plus hydrogen peroxide; (b) K12 minus hydrogen peroxide; (c) DH5a minus hydrogen peroxide; (d) DH5a plus hydrogen peroxide.

Our experiments enabled us to estimate that an individual *E. coli* cell secretes approx. 30 fMet-Leu-Phe molecules/min and this increases to about 120 upon stimulation with u.v. light. This observed 4-fold increase in fMet-Leu-Phe production is somewhat less than would be predicted from the 13-fold increase in UmuD production reported for the fully derepressed SOS operon [25]. Our observation that the *RecA*⁻ strain (DH5a) was a significantly lower producer of fMet-Leu-Phe than the other strains of *E. coli* is consistent with the suggestion that the basal production of fMet-Leu-Phe is dependent on RecA. However, it is possible that this *RecA*⁻ strain is also mutated in other genes which affect UmuD processing, or that there are other sources of fMet-Leu-Phe from high-turnover signal peptides which cannot be visualized by Western blots.

Hydrogen peroxide and u.v.-irradiation treatment of wild-type *E. coli*, but not *RecA*⁻ strains, promoted increased secretion of fMet-Leu-Phe. This effect was inhibited by addition of chlor-

Figure 7 The secretion of fMet-Leu-Phe-equivalent immunoreactivity after non-lethal exposure to u.v. irradiation

E. coli K12 cells were exposed to u.v. irradiation and then the secretion of fMet-Leu-Phe (fMLP) determined as described in the Materials and methods section. Numbers above histograms are fractional growth rates (e.g. control = 10, 10 s exposure = 0.81 indicating impaired growth rate).

amphenicol, indicating it did not simply arise by release of a preformed pool of the peptide.

The relationship between oxidant-induced activation of SOS genes and production of the chemotactic peptide in *E. coli* suggests a possible hypothesis to explain the biological significance of the fMet-Leu-Phe receptor on mammalian leucocytes, particularly neutrophils. This to some extent anticipates that production of fMet-Leu-Phe or closely similar peptides is fairly ubiquitous among other bacterial genera. There is limited evidence that this is so [19,26,27] but no direct evidence yet that chemotactic peptides in other organisms arise by similar mechanisms (e.g. SOS activation) to those reported here for *E. coli*. Notwithstanding this deficiency in experimental data, we propose that host phagocytes respond to fMet-Leu-Phe and closely related peptides because they are generated by bacteria under oxidant stress. This would occur when invading organisms gain access to tissues where pO₂ levels are high. Even relatively aerotolerant organisms with oxidant-scavenging enzymes such as superoxide dismutase, catalase and peroxidases are susceptible to DNA damage at high pO₂ levels, whereas anaerobic or microaerophilic organisms of the kind usually inhabiting the lower intestine would be especially vulnerable [28,29]. Thus increased fMet-Leu-Phe production by, for example, *E. coli* may signal a change in the organism's biological status from commensal, such as in the intestinal lumen, to pathogen in tissue sites such as the kidney. Furthermore phagocytes, which may release oxidants extracellularly, could further stress bacteria in tissue sites leading to enhanced chemotactic peptide production and further leucocyte recruitment.

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