The TolC protein of *Escherichia coli* K12 is synthesised in a precursor form

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We examined the biosynthesis of the TolC protein of *Escherichia coli* Kl2 in a pulse–chase experiment, followed by immunoprecipitation with anti-TolC antibody and SDS–PAGE of the immunoprecipitate. This showed that TolC protein was originally synthesised in a precursor form (M_r 54 500) which could be chased into the mature form (M_r 52 000). DNA sequencing of a portion of the cloned *tolC* gene showed that the N-terminus of the mature rotein was preceded by a typical signal sequence of 22 residues (M_r 2542). The initiator Met was preceded by a Shine–Dalgarno sequence, with the correct spacing.

TolC protein *Signal sequence* *E. scherichia coli outer membrane* *colicin El* *DNA sequencing* *OmpF protein*

1. INTRODUCTION

The *tolC* gene of *Escherichia coli* K12 is necessary if the major outer membrane proteins OmpC, NmpC and protein 2 are to be present in normal amounts [1]. Strains mutant in the *tolC* gene show hypersensitivity to various dyes and detergents, and are also tolerant to colicin E1 [2]. The *tolC* gene has been cloned, and examination of the cloned product both in minicells and intact bacteria showed that the TolC protein was an outer membrane protein of M_r ~52000 [3,4].

Here, we report that the *tolC* gene product is synthesised in a form which has app. M_r ~54500, which can be chased into a product of the same M_r as the mature protein. DNA sequencing of a portion of the cloned *tolC* gene showed that the N-terminal amino acid of the mature protein (Glu) was not immediately preceded by a codon for a Met residue, but that Met codons occurred 22 and 24 amino acids N-terminal to the N-terminus of the mature protein. We suggest that the 22 residue stretch of amino acids is originally attached to the mature protein, and that it is a signal peptide, being removed proteolytically as or after the protein becomes localised in the bacterial membrane [5].

2. MATERIALS AND METHODS

2.1. *Materials*

Enzymes active on DNA were from Boehringer. The source of the sequenced DNA was the plasmid pPR42 [3]. Synthetic primer for M13 sequencing was from P-L Biochemicals. L-[^{35}S]Methionine was from Amersham (Bucks) while [α-^{32}P]dCTP, for sequencing, was from BRESA (Adelaide, SA).

2.2. *DNA sequencing*

The chain termination method was used [6]. The plasmid pPR42 [3] was cut with *Pst*I, and the 1.1 kilobasepair *Pst* fragment which includes some of the *tolC* gene [3] was purified on an agarose gel. This fragment was cut with *Sau*3A, and the pieces ligated into *Bam*HI-cut replicative form of phage M13mp7 [7]. Recombinant phage DNA was sequenced [7].

2.3. *Pulse–chase experiments*

The pulse–chase experiments were designed to label short-lived forms of the TolC protein and chase these forms into mature protein. Two strains were used. The strain P2715 was Ab1133 of A.L. Taylor (F$^-$, *thr-1, leu-6, proA2, lacY1, supE44,*

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galK2, his-4, rpsL31, xyl-5, mtl-1, argE3, thi-1, ara-14, λ^-) transformed with pPR42. The strain P602 was a *tolC* mutant of AB1133 (*tolC203*) [8]. Cells were grown at 37°C in M9 medium supplemented with the necessary growth factors. Glucose (0.5%, w/v) was the carbon source.

Exponential stage cells (10 ml of A_{600} = 0.6) were pelletted and resuspended in 1/10 vol. original medium. After 5 min pre-incubation at 25°C, L-[^{35}S]methionine was added to 50 μCi/ml. The pulse time was 10 s at 25°C, and samples were taken at various times after addition of cold L-methionine (0.2 M) to 20 mM. Each sample contained 5×10^8 cells, and was added to crushed ice (~100 μl) containing 0.2% (w/v) NaN_3. Cells were pelletted in the Microfuge at 4°C and resuspended in SDS buffer (100 mM Tris, 5 mM EDTA, 0.5% (w/v) SDS, 0.02% NaN_3, pH 7.4) (30 μl), then boiled for 3 min.

2.4. *Immunoprecipitation of labeled proteins*

To obtain antiserum to the TolC protein, purified protein [4] (0.75 mg) was suspended in Freund's complete adjuvant and injected into a rabbit. Booster shots (0.5 mg × 2) followed at 14-day intervals. Immunoprecipitation of labeled proteins was done according to Pittard (personal communication).

SDS-solubilised cells (2×10^8; section 2.3) were diluted 10-fold with Triton buffer [100 mM Tris, 1 mM EDTA, 0.02% (w/v) NaN_3, 1% (w/v) Triton X-100 (pH 7.4)]. Antiserum (5 μl) was added and the solution incubated at 37°C for 1 h. Affinity-purified goat-anti-rabbit IgG (20 μl 4 mg/ml, donated by P. Ey) was added and, after 3 h at 37°C, the precipitate was collected by centrifugation (Microfuge, 15 s). The precipitate was washed twice with Triton buffer, and then twice with Tris buffer [100 mM Tris, 1 mM EDTA, 0.02% (w/v) NaN_3 (pH 7.4)] followed by suspension in buffer containing SDS and mercaptoethanol (30 μl) [9] prior to gel analysis.

2.5. *Polyacrylamide gel electrophoresis*

Samples were boiled for 3 min and electrophoresis proceeded in 11% (w/v) polyacrylamide gels containing 0.2% (w/v) SDS [9]. The dried gels were autoradiographed, and the band intensities quantitated with a Helena Laboratories Scanner.

3. RESULTS AND DISCUSSION

3.1. *TolC protein antiserum precipitates a higher-M_r precursor of the TolC protein*

Strain P2715 was labeled with L-[^{35}S]methionine for 10 s and chased for various times. The SDS-solubilised cells were subjected to immunoprecipitation with antiserum to TolC protein followed by addition of goat-anti-rabbit IgG (sections 2.3 and 2.4). The immunoprecipitates were viewed on polyacrylamide gels (fig.1). At M_r 50000–56000, two labeled bands appeared (fig.1).

Although the kinetics of processing may be complex, the overall effect seen in the chase was the decrease of the higher-M_r band from 76% of total label at 15 s chase to 1% at 300 s of chase, while the lower-M_r band rose from 24% of total label at 15 s to 99% of total label over the same period. This is evidence that the higher-M_r band is a precursor of the lower-M_r band.

As a control of the specificity of the TolC antiserum used, strain P602 (*tolC*) was examined in a pulse–chase experiment as above. No labeled bands could be detected on gels of im-

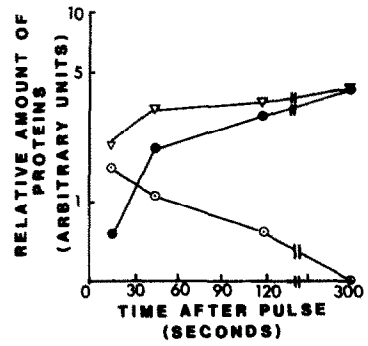

Fig.1. Pulse–chase experiment to show that the TolC protein is initially made in a high-M_r form. Strain P2715 (carrying a high copy number *tolC*$^+$ plasmid) was pulsed with L-[^{35}S]methionine for 10 s and chased for varying times with cold L-methionine. Solubilised cells were immunoprecipitated with antiserum to TolC protein and goat-anti-rabbit IgG. The immunoprecipitates were solubilised, examined by PAGE, and subjected to autoradiography (upper). The gel region containing polypeptides of M_r 50000–56000 was scanned (lower); P, M, proposed precursor and mature TolC protein, respectively. The open vertical arrows indicate the peak corresponding to precursor TolC protein; (1) 15 s chase; (2) 45 s; (3) 120 s; (4) 300 s.

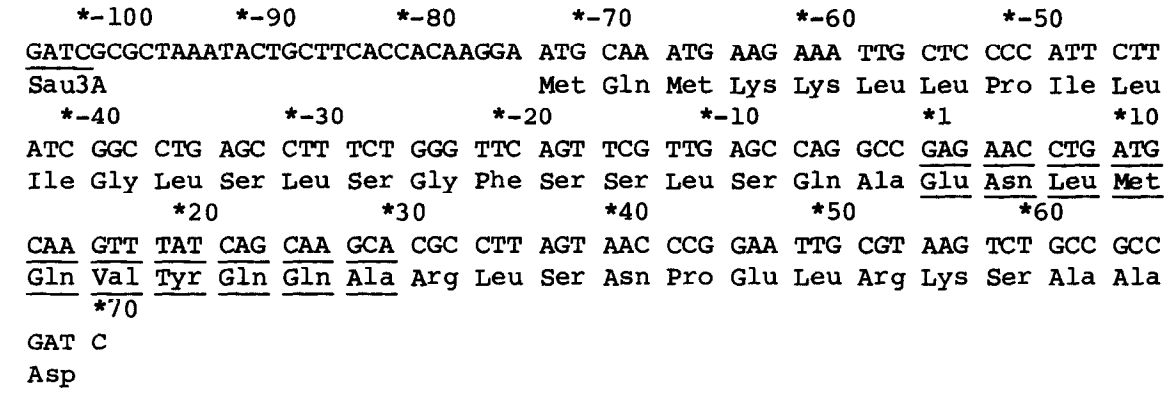

Fig.2. The sequence of a 173 basepair *Sau*3A fragment containing the TolC protein N-terminus and the signal sequence. For cloning and sequencing details, see the text. The position 1 is the first G of the GAG (Glu) codon which is the first amino acid of the mature protein. The first 10 amino acids of the mature protein [4] are underlined.

munoprecipitates (not shown). The *tolC* mutation therefore results in the disappearance of both the higher- and lower-M_r labeled bands of fig.1, in line with the idea that the higher-M_r band is a precursor of the lower-M_r band.

3.2. *DNA sequencing of the N-terminal area of the* tolC *gene*

As part of our preparation for sequencing the *tolC* gene, we isolated a 1.1 kilobasepair *Pst*I fragment from pPR42 [3], which contained part of the *tolC* gene, as shown by transposon-insertion mutagenesis [3]. This fragment was cut with *Sau*3A, and the *Sau*3A fragments cloned in M13mp7. A 173 basepair *Sau*3A fragment contained a sequence corresponding to the 10 N-terminal amino acids of the mature protein [4] (fig.2). The TolC protein sequence reads from left to right in the map of pPR42 [3]; the first G of the GAG (Glu) codon which is the first amino acid of the mature protein lies 255 nucleotides to the left of the first C of the *Pst*I cut in the *tolC* gene.

The GAG (Glu) codon which codes for the first amino acid of the mature protein is not preceded by a Met codon; such codons (ATG) occur 66 and 72 bases upstream from the GAG (fig.2). The AGGAA (−76 to −72) (fig.2) may be a Shine-Dalgarno sequence [13]; the GG of this sequence is separated from the ATG codons by 1 and 7 bases. Since the spacing between the Shine-Dalgarno GG and the first base of the initiation codon is typically 5–9 bases [14], this may indicate that the ATG at position −66 is actually the initiation codon. This would give a signal sequence of 22 residues, of M_r 2542.

The signal sequence described possesses features frequently observed in bacterial signal sequences [5]. A pair of basic amino acids (Lys–Lys) occurs near the N-terminus. There is a stretch of 8 hydrophobic residues (Leu–Leu–Pro–Ile–Leu–Ile–Gly–Leu) which contains a Pro residue. The amino acid next to the cleavage site (Ala) has a one-carbon side chain.

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