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Export of a Protein into the Outer Membrane of *Escherichia coli* K12

Stable Incorporation of the OmpA Protein Requires Less than 193 Amino-Terminal Amino-Acid Residues

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The cloned *ompA* gene encoding the major outer membrane protein OmpA of *Escherichia coli* has been shortened *in vitro* by exonuclease digestion from the end corresponding to the CO_2H terminus of the protein. Nine derivatives were identified which still possessed substantial parts of the *ompA* gene and one was constructed which had suffered a small deletion early in the gene. Gene fragments encoding NH_2-terminal OmpA sequences of 45, 133, 193, and 227 residues of the 325 amino acids of OmpA were examined in detail at the DNA level and for OmpA protein fragments synthesized. The latter two fragments were incorporated into the outer membrane and all known functions of the OmpA protein were expressed whereas the fragment with 133 OmpA-specific residues was not stably incorporated into this membrane.

In all cases where OmpA functions were observed, an OmpA-specific polypeptide of M_r 24000 was found in cell envelopes, regardless of the size of the residual *ompA* sequences and of the fused coding sequences in the vector DNA. Pulse-label experiments revealed larger initial translation products, most of which were degraded to the protein of M_r 24000. The 133-residue OmpA fragment was also detected but proved to be entirely unstable.

It is argued that the OmpA protein consists of two domains and that the NH_2-terminal moiety from residues 1 to about 180 represents the membrane domain of the polypeptide. Therefore, the loss of about 50, possibly less, CO_2H-terminal residues from this domain suffices to interfere with stable incorporation into the outer membrane.

The OmpA protein (formerly called protein II* by us [1]), is one of the abundant polypeptides of the *Escherichia coli* outer membrane (for a recent review see [2]). The protein consists of 325 amino acid residues [3] and is synthesized as a precursor, the pro-OmpA protein, with a 21-residue signal sequence [4–6]. We have cloned the structural gene *ompA* [7] and shown that it is possible to subclone into plasmid pBR322 [8] a *Bam*HI fragment, from a mutant *ompA* gene, carrying the codons for the 227 NH_2-terminal residues of the protein. It was found that the corresponding protein fragment, missing 98 CO_2H-terminal amino acid residues, is strongly produced and is incorporated into the outer membrane [9]. Furthermore this OmpA′ protein still serves as a receptor for OmpA-specific phages [10, 11].

We have now asked to what extent the *ompA′* gene can be further shortened without impairing outer membrane incorporation of the resulting protein fragments. Here we show that more than 133 NH_2-terminal residues of the mature protein are required and that 193 such residues suffice for a stable incorporation into this membrane.

Enzymes. Pronase (EC 3.4.24.4 + 3.4.21.4); trypsin (EC 3.4.21.4); DNA polymerase I (EC 2.7.7.7); T4 polynucleotide ligase (EC 6.5.1.1); T4 polynucleotide kinase (EC 2.7.1.78); S1 nuclease (EC 3.1.30.1); restriction endonucleases: *Bam*HI (EC 3.1.23.6), *Eco*RI (EC 3.1.23.13), *Hae*III (EC 3.1.23.17), *Hin*fI (EC 3.1.23.22), *Hpa*I (EC 3.1.23.23), *Pvu*II (EC 3.1.23.33), *Rsa*I (EC 3.1.23.–), *Sal*I (EC 3.1.23.37).

MATERIALS AND METHODS

Nomenclature

The protein in question is called the OmpA protein and its gene *ompA*. The *Bam*HI-generated fragment of the gene encoding residues 1–227 is designated *ompA′* and its product the OmpA′ protein. All amino acid residue numbers refer to the mature OmpA protein and not to the pro-OmpA protein.

General Methods

The strains used are listed in Table 1. All plasmids constructed were derived from pTU302 [9] which is a derivative of pBR322 [8]. Cells were grown at 37 °C in L-broth supplemented with ampicillin (20 μg/ml) when they carried plasmids. For detection of constitutive expression of the *lac* operon (caused by the presence of the adaptor AD3 present on pBR322) minimal-glucose medium containing X-gal (chlorobromoindolyl galactoside, Sigma) was used [12].

For DNA sequencing endonuclease fragments were 5′ end-labelled with [γ-^{32}P]ATP (> 3000 Ci/mmol; Amersham) and T4 polynucleotide kinase and further processed as described by Maxam and Gilbert [13]. T4 DNA ligase, DNA polymerase I [14], exonuclease III [14], T4 polynucleotide kinase, S1 nuclease [15], restriction endonucleases [16] and BAL31 were isolated as described or purchased from commercial

Table 1. *Strains of* Escherichia coli *used*
Strain UH201-3 was derived from UH201: a spontaneous *str* mutant was made *his* (diethylsulfate mutagenesis and penicillin selection); a cross with KL16-99 (*thi*, *recA*, *Hfr* [45]), selection for *str*, *his*$^+$, and screening for *recA* by ultraviolet irradiation yielded UH201-3. Nomenclature is according to Bachman and Low [46]

Strain	Origin	Genotype
UH100	W620 [10]	*thi*, *pyrD*, *gltA*, *galK*, *str*, *trp*, *recA*, *ompA*
UH201	JC6650 [43]	F$^-$/*lac*, *supF*, *ompA*
UH201-3	UH201 (this study)	*lac*, *supF*, *str*, *recA*, *ompA*
C600	Cold Spring Harbor Collection	rk$^-$, mk$^+$
JC5484	P. A. Manning [44]	F′ *lac*$^+$/*his*, *trp*, *lac*, *tonA*, *tsx*

sources (Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs) and used as recommended by the manufactures. Ligation buffer is 50 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 1 mM EDTA.

Adaptor Fragment AD3

Adaptor fragment AD3 is a 43-base-pair *Bam*HI fragment which was constructed by blunt-end ligation of a synthetic *lac* operator fragment [17] containing two *Eco*RI restriction sites and a commercial *Bam*HI decanucleotide linker (Ch. Kurz and H. Schaller, unpublished). Its nucleotide sequence (see Fig. 3) contains several stop codons in either orientation. The fragment was cloned and amplified in plasmid pBR322 [8] and isolated by cleavage with *Bam*HI followed by preparative gel electrophoresis on a 6% polyacrylamide gel. Clones bearing plasmids containing the AD3 adaptor fragment appear as blue colonies on X-gal medium since a high copy number of the *lac* operator fragment confers constitutive expression of the *lac* operon to transformed *Escherichia coli* cells which leads to hydrolysis of the indicator dye X-gal [17].

Construction of Plasmid pOMPA21

Plasmid pTU302 (8 µg, 2 pmol), linearized by restriction endonuclease *Sal*I, was dissolved in 40 µl of 100 mM potassium phosphate, pH 7.0, 100 mM KCl, 7 mM $MgCl_2$, 3 mM dithiothreitol and incubated at 30 °C with 100 units of *E. coli* exonuclease III. Samples of 10 µl were removed at 10-min intervals and undigested DNA was precipitated with 25 µl of ethanol. Precipitates were dried, redissolved in 30 µl of 30 mM sodium acetate pH 4.6, 280 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol and incubated with 0.05 unit of S1 nuclease. After 5 min at 37 °C, nuclease digestion was terminated by the addition of 30 µl of 0.1 M Tris pH 8.0, 10 mM EDTA. Each sample was extracted with an equal volume of phenol and desalted by chromatography on Sephadex G-75 in 10 mM ammonium bicarbonate followed by lyophilization. For simultaneous gap filling and ligation, the samples were dissolved in 10 µl of ligation buffer containing all four deoxyribonucleoside triphosphates (20 µM each) and incubated at 15 °C for 10 h with *E. coli* DNA polymerase I [14] and T4 DNA ligase. Samples of 2 µl were used to transform *E. coli* C600. Plasmids from ampicillin-resistant colonies were analysed for reduced size, for loss of one of the two *Bam*HI cleavage sites and for the presence of the *Hin*fI site at position 781 (see Fig. 1).

Construction of Plasmids pOMPA21-2 to pOMPA21-22

Plasmid pOMPA21 (1 pmol) linearized by cleavage with endonuclease *Bam*HI was incubated at 37 °C with exonuclease BAL31 (6 units) in 50 µl of 20 mM Tris pH 8.1, 600 mM NaCl, 12 mM $MgCl_2$, 12 mM $CaCl_2$, 1 mM EDTA. Aliquots of 16 µl were withdrawn after 2 min, 5 min and 10 min respectively. These were combined, phenol-extracted and desalted on Sephadex G-75. One sixth of the DNA mixture (0.15 pmol) was mixed with 0.1 pmol of AD3 adaptor fragment and simultaneously filled in and ligated as described. The ligation mixture was used to transform competent *E. coli* C600 cells and ampicillin-resistant colonies were analysed on X-gal plates. Plasmid DNA from 28 blue clones was isolated using a minilysate method [18], and the approximate extent of the deletions determined by restriction analysis with *Eco*RI and *Hin*fI.

Construction of Plasmid pOMPA21-30

Plasmid pTU302 DNA (2 pmol) was digested with *Hpa*I and *Bam*HI, then purified by phenol extraction and chromatography on a Sephadex G-75 column in 10 mM ammonium bicarbonate. The lyophilized fragments were simultaneously filled in and ligated and the mixture used directly for transformation.

Determination of Deletion End Points and of Fusion Sequences

The exact position where the shortened *ompA′* sequences were fused to the AD3 fragment was established in 14 plasmids by determining the positions of the dG residues relative to the *Eco*RI sites in the adaptor fragment. Plasmid DNA was 5′ end-labelled at the three *Eco*RI cleavage sites as described by Maxam and Gilbert [13], cleaved with *Hin*fI and the resulting fragments separated on a 6% polyacrylamide gel. Usually four labelled DNA fragments were obtained: two of constant size from the vector (1000 base pairs, position 4362 – 3362) and from inside the adaptor (29 base pairs, see Fig. 3), and two of variable length containing the deletion end-points next to their labelled *Eco*RI termini. These latter fragments were isolated, subjected to partial dG-specific chemical cleavage and analysed on a DNA sequencing gel as described [13].

Junction sequences were determined from plasmids pOMPA21-11 and pOMPA21-16 after 5′ end-labelling of the *Rsa*I sites at positions 1483 and 1664 in the *ompA′* gene, respectively. In both cases restriction endonuclease *Hae*III was used for secondary cleavage at position 297 as depicted in Fig. 2. The junction sequence in plasmid pOMPA21-30 was determined from the *Hae*III site at position 1181 after 5′ end-labelling and secondary cleavage with *Bam*HI.

Analysis of Cell Envelopes

Cells for labelling were grown at 25 °C in M9 minimal salts medium [12] enriched with L-broth (2%) and supplemented with glucose (0.4%), glutamate (100 µg/ml), uridine (50 µg/ml), tryptophan (50 µg/ml) and, in the case of plasmid-bearing strains, ampicillin (20 µg/ml). The addition of L-broth

was required because without it the strains stopped growing at a density of 2×10^8/ml. The bacteria were harvested by centrifugation, at a cell density of 5×10^8/ml, then washed twice with M9 medium. After 10-fold concentration in unenriched M9 medium (supplemented as above) the cells were shaken for 1 h at 25 °C. They were then labelled with [^{35}S]-methionine (1000 Ci/mmol; Amersham) at a concentration of 20 µCi/ml. Labelling was terminated by rapidly mixing 80 µl cells with 20 µl of an ice-cold solution containing 300 mM Tris/HCl pH 6.8, 30% glycerol, 12% sodium dodecylsulphate, and 20% mercaptoethanol. These suspensions were boiled for 5 min and used directly for sodium dodecyl sulphate/polyacrylamide gel electrophoresis [19]. The gels were subjected to the immunoreplica technique described by Showe et al. [20] with some modifications. Molten 1% agarose (Standard Low-M_r, Bio-Rad) 6.3 ml in 10 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulphate was mixed at 42 °C with 1.3 ml rabbit anti-OmpA serum [21]. The mixture was poured onto a glass plate on which an aqueous solution of 0.2% agarose had been dried. The electrophoresis gel was placed onto the agarose gel, overlaid with a glass plate and the assembly left for 16 h in a moist atmosphere at room temperature. The agarose gel was washed for 24 h with three changes (700 ml each) of 10 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.01% sodium dodecyl sulphate. It was then soaked for 30 min in 1 M sodium salicylate [22], dried at 37 °C, and fluorographed using 'preflashed' films [23]. The exposure time was about 4 days.

Function of OmpA' Proteins

A crude preparation of colicin L-JF246 (NaCl extract) was prepared from *Serratia marcescens* JF246 (kindly donated by J. Foulds) and sensitivity to this colicin was tested as described [24]. Strains to be tested as recipients in conjugation were grown to stationary phase. The F'*lac*$^+$ donor JC5484 was grown to about 2×10^8 cells/ml. The mixture of donor (5×10^7/ml) and recipient (10^8/ml) was incubated for 40 min at 37 °C and appropriate dilutions were plated onto McConkey lactose agar (Difco) containing streptomycin (200 µg/ml).

RESULTS

Construction of Plasmid pOMPA21

Earlier work on *ompA* structure and function had been carried out with plasmid pTU302 [9]. As depicted in Fig. 1 this plasmid contains about 800 base pairs of non-essential *Escherichia coli* DNA upstream from the *ompA'* gene. To facilitate the manipulation of the cloned gene it was desirable to delete most of these additional DNA sequences from pTU302. For this purpose the plasmid was opened at the unique *Sal*I site in the vector and shortened by successive treatments with *E. coli* exonuclease III and S1 nuclease. From the resulting deletion variants one, pOMPA21, proved to be well suited for the further modification of the *ompA'* gene. As indicated in Fig. 1 the end points of deletion have been mapped between nucleotide positions 680 and 533 as deduced from the *Hin*fI restriction maps of pTU302 and the presence of a new 260-base-pair fragment at the vector-insert junction. The new plasmid retains the intact signals and the coding sequence for *ompA'* expression but contains only one of the *Bam*HI sites and one of the *Hpa*I sites that were present in the parent plasmid. Thus, these unique sites could be used in the following study as specific targets to open and manipulate the *ompA'* nucleotide sequence. No difference in the levels of expression of the *ompA'* genes carried by pOMPA21 and pTU302 was observed (data not shown).

Further Shortening of the ompA' *Gene*

Plasmid pOMPA21 was opened at the remaining *Bam*HI site (position 1780), shortened by digestion with exonuclease BAL31 and religated under conditions generating blunt ends. To facilitate the isolation and analysis of the expected deletion mutants the 43-base-pair adaptor fragment, AD3, was included in the ligation mixture. Clones harboring plasmids carrying this DNA insert can be recognised on X-gal plates as blue colonies (see Materials and Methods). In addition, the newly formed junction sequences can be analysed easily from its internal *Eco*RI cleavage sites (see Fig. 3).

Sequence analysis for the distribution of a single base (guanine) sufficed to locate the end points of the deletions in the known nucleotide sequence of the parent plasmid. Plasmids from 14 blue colonies were characterized by this method. Nine of these were found to retain a substantial part of the *ompA'* gene (see Table 2), two had lost all of *ompA'* including the *ompA* promotor at position 860–900 [25], and three isolates had lost all *E. coli* DNA sequences.

None of the clones was found to terminate within the sequence coding for the first 130 amino acids of the OmpA' protein. A deletion was therefore constructed by fusing the *Hpa*I site at position 1232 to the *Bam*HI site at position 1780 of plasmid pOMPA21 (Fig. 1). The plasmid was digested with both endonucleases then simultaneously treated with DNA polymerase I and DNA ligase and the mixture used to transform strain UH100. Plasmids from 20 ampicillin-resistant clones were analyzed by cleavage with the appropriate restriction endonucleases and five of these proved to have lost the *Hpa*I-*Bam*HI fragment. Although the remainder still possessed this fragment it was found in 12 cases that the *Hpa*I site at position 1232 had been lost. This indicated that the nucleotide sequence of the *ompA'* gene had been changed at this site during reinsertion of the '*Hpa*I'-*Bam*HI fragment.

Function of OmpA' Fragments

The *ompA'* gene used in this study carries a TAG stop codon corresponding to amino acid residue 7 of the protein and its products are only expressed well in the presence of an efficient suppressor [9]. Plasmids listed in Table 2 were therefore transformed into an *ompA* mutant strain carrying *supF* (UH201-3). Because the OmpA protein can serve as a phage receptor, we tested the resulting strains for sensitivity to phage K3 and its extended host range mutant, K3h1 [26]. The latter phage can infect *ompA* mutants expressing the protein at very low levels [26,27]. The results are included in Table 2 and show that *ompA'* fragments encoding at least 193 amino acid residues confer phage sensitivity to the host while the two fragments coding for 45 and 133 residues can no longer do so.

The plasmids from the 20 clones obtained from the experiment in which the *Hpa*I site position 1232 was fused to the *Bam*HI site at position 1780 were also transformed into strain UH201-3. As expected from the behavior of UH201-3 carrying pOMPA21-11, those that had lost the *Hpa*I-*Bam*HI fragment could not confer phage sensitivity. Furthermore, the 12 plasmids lacking the *Hpa*I site alone

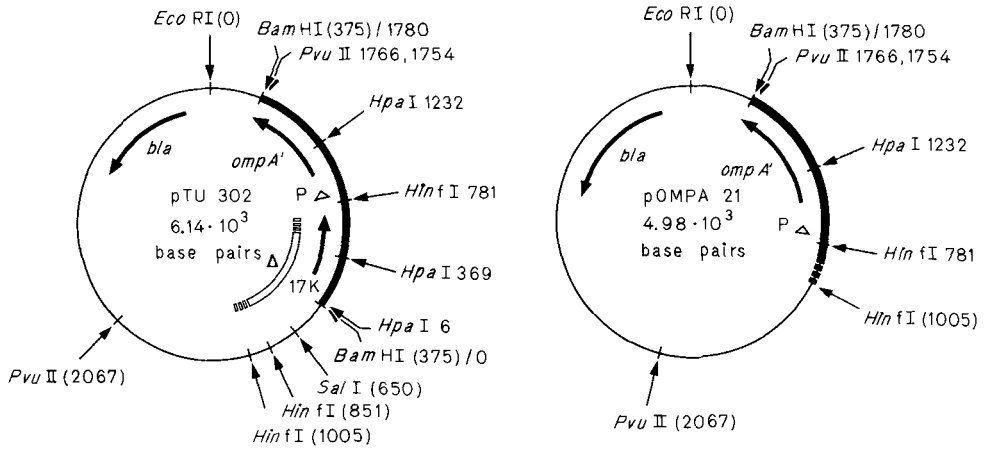

Fig. 1. *Structure and relevant restriction sites in plasmids pTU302 and pOMPA21.* The heavy segment represents *Escherichia coli* DNA from the *ompA* region with map positions according to [4]. The thin part of the circles represents pBR322 sequences and corresponding map positions [32] are noted in parentheses. Arrows indicate the products of the following genes: *bla*, β-lactamase from the vector; *ompA'*, a functional fragment from *ompA* coding for 227 NH_2-terminal amino acids of the OmpA protein [3] and 17K a hypothetical gene [4]. The ompA promotor [25] is denoted by P*Δ*. A DNA segment that has been deleted from pTU302 to yield pOMPA21 is indicated by an open line marked with *Δ*. It should be noted that expression of *ompA'* is unaffected by the deletion of the gene for the hypothetical 17000-M_r protein

Table 2. *Deletions in* ompA *fragments*

Plasmid	Deletion and end point, base pair position [4]	OmpA amino acid residues still coded for[a]	Resistance (R) or sensitivity (S) to phages K3 or K3h1
pTU302/pOMPA21	1780	227	S
pOMPA21-15, -18	1766	222	S
pOMPA21-5	1765	222	S
pOMPA21-23	1758	219	S
pOMPA21-6	1749	216	S
pOMPA21-2	1740	213	S
pOMPA21-16, -22	1678	193	S
pOMPA21-11	1499	133	R
pOMPA21-30	1234	45[b]	R

[a] Not including the signal sequence.
[b] See following text.

Table 3. *Action of OmpA protein and OmpA' fragments in conjugation*
The donor used in all cases was strain JC5484

Recipient strain	F'*lac*⁺ transconjugants
	% of *lac*
JC6650 (*ompA*⁺)	44
UH201-3 (*ompA*)	0.1
UH201-3 with pTU302	30
UH201-3 with pOMPA21-16	25
UH201-3 with pOMPA21-11	0.3

(and among these pOMPA21-30; see Table 2) were also unable to generate phage sensitivity. The remaining three plasmids could do so, thereby indicating that they contained the restored *ompA'* gene.

Besides phage receptor activity, the protein is required for the action of colicin L-JF246 [28] and it serves as a mediator, on the side of the recipient, in F-dependent conjugation [11, 29–31]. The areas of the protein necessary for these three functions are different [31]. We have tested derivatives of strain UH201-3 harboring all plasmids listed in Table 2 for their sensitivity to colicin L-JF246. The strains carrying pOMPA21-11 or pOMPA-21-30 were as highly tolerant to the colicin as UH201-3 without a plasmid. The colicin titers obtained when UH201-3 derivatives bearing all the other plasmids were used are the same as that measured with the *ompA*⁺ parent of strain UH100 (data not shown). We have also tested the efficiency of transfer of a F'*lac*⁺ factor into the relevant strains. Table 3 shows that plasmid-carrying strains which exhibit phage and colicin sensitivity were fully efficient recipients in conjugation.

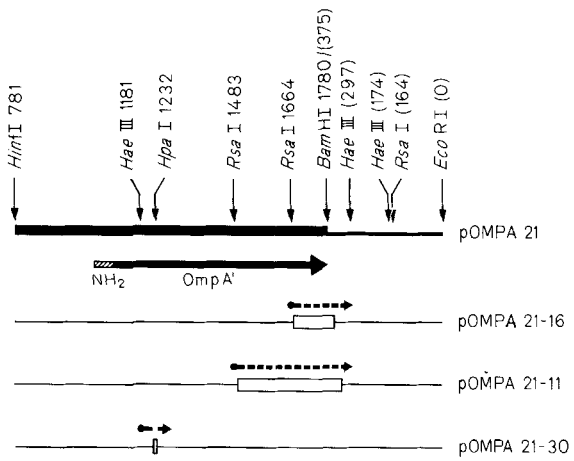

Fig. 2. *DNA sequencing strategies.* The strategies used to sequence deletion end points in pOMPA21-11, pOMPA21-16 and pOMPA21-30 are shown. The open boxes represent the deletions and the arrows the sequence information obtained. The position of the labelled ends is indicated (●). Depicted in the upper portion is the organisation of the *ompA'* gene in pOMPA21 (see Fig. 1). The OmpA' protein and its signal sequence are represented by the solid and hatched parts, respectively, of the large arrow. Relevant restriction sites are shown by vertical arrows

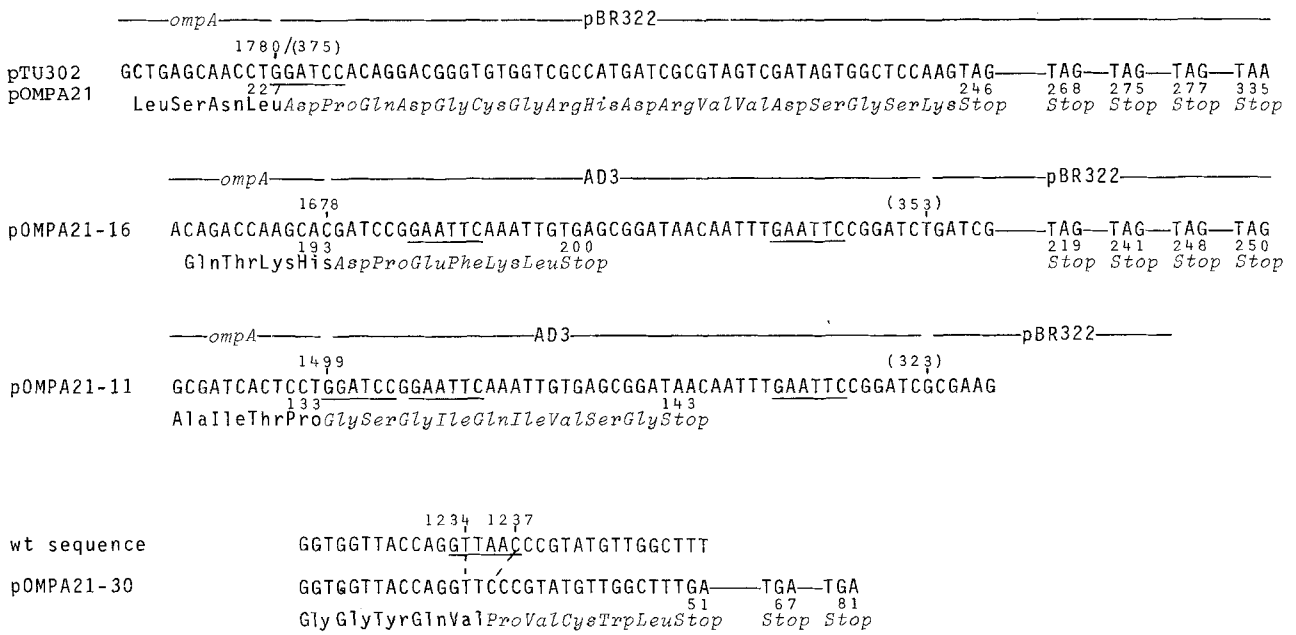

Fig. 3. *Junction sequences covering deletion end points.* The sequences of the deletion end points from pOMPA21-11, pOMPA21-16 and pOMPA21-30 together with the junction sequence around the *BamH*I site in pTU302/pOMPA21 are shown. The positions of the last nucleotide in OmpA and of the first nucleotide in pBR322 are indicated. The d representing deoxy and the hyphens representing phosphodiester linkages have been omitted. The numbers at amino acid residues or stops correspond to residue numbers of the OmpA protein [3]. Recognition sequences for relevant restriction endonucleases are underlined: *Bam*HI, d(G-G-A-T-C-C); *Eco*RI, d(G-A-A-T-T-C); *Hpa*I, d(G-T-T-A-A-C). It is of interest to note that the exonuclease BAL31 has worked rather asymmetrically: in pOMPA21-16 it cut into *ompA'* by 102 and into the vector by 23 base pairs whereas in pOMPA21-11 281 bases were lost from *ompA'* and 52 from the vector

DNA Sequence Analysis of ompA' *Fusions from Plasmids pOMPA21-11, pOMPA21-16, and pOMPA21-30*

Because the orientation of the adaptor fragment and the precise site of its fusion to pBR322 sequences [32] were unknown, we determined the exact nucleotide sequence in this region of plasmids pOMPA21-11 and pOMPA21-16. This information was desirable in order to predict which protein fusion products could be expected. The sequencing strategies are outlined in the Materials and Methods and in Fig. 2 and the nucleotide sequences obtained are shown in Fig. 3. Furthermore, as it was uncertain what had occurred in those plasmids which had lost the *Hpa*I site at position 1232, we sequenced a representative, pOMPA21-30. The *ompA'* gene carried by this plasmid was found to have suffered a deletion of two dA · dT pairs at the original *Hpa*I site. It is not clear whether this loss was due to the 5′ exonuclease activity of the DNA polymerase I which was present in the ligation mixture or to a contaminant in the commercial enzyme preparation. No analogous loss of terminal nucleotides was observed at the filled in ends of the AD3 adaptor in all 14 cases analysed.

PRODUCTS OF THE PARTIALLY DELETED *ompA'* GENES

Products Expected

Using the known sequences of the *ompA* gene [4, 25] and of plasmid pBR322 [32], the DNA sequences determined allowed us to find the translational stop codons following the residual *ompA'* sequences. These stops are also shown in Fig. 3 and the M_r values of the corresponding hypothetical polypeptides are shown in Table 4. TAA stops are effective in strain UH201-3. TAG codons are supressible by *supF* but when two or more such codons occur in close proximity they should constitute an efficient translational termination signal. TGA stops are leaky [33] but we do not know how leaky this stop is in strain UH201-3 or whether it carries a TGA suppressor.

Other uncertainties exist concerning the size of the proteins expected to be made. Firstly, the sizes of all these potential polypeptides have been calculated without taking into account the 21-residue signal sequence. Thus processing is assumed to occur although this may not be the case. Secondly, the OmpA protein exhibits an aberrant electrophoretic mobility. It migrates as a 33000-M_r species while its true M_r is 35159 [3]. On the other hand, a pronase fragment missing part of the CO_2H terminus can be isolated; this fragment electrophoretically migrates as a 19000-M_r species and its chemically calculated M_r is 19282 [3]. From several observations (unpublished data) it is likely that another proteolytic fragment generated by trypsin exhibits the correct M_r of 24000 [34]. We do not know exactly which part of the polypeptide is responsible for the aberrant mobility; thus a safe correlation between deduced and observed M_r values is not possible for products with M_r larger than about 24000.

Products Found

Cell envelopes from UH201-3 derivatives carrying the various plasmids listed in Table 2 were analysed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis and the proteins visualised by staining with Coomassie blue. Representative examples are presented in Fig. 4. Surprisingly, in all cases where phage sensitivity was observed a protein fragment was found that was identical in size, $M_r = 24000$, to that produced by the parent plasmid, pTU302. The

Table 4. *Products of partially deleted* ompA *genes*
The M_r of the OmpA-specific sequences still encoded and the possible translation products do not take into account the 21-residue signal sequence (M_r 1936); the former are calculated values; the latter are approximate values arrived at by using an average M_r of 110 per amino acid residues. The predominant species found are printed in bold-face type; the numbers at the stop codons correspond to amino acid residue numbers of the OmpA protein (cf. Fig. 3)

Plasmid	M_r of OmpA-specific sequences still encoded (hypothetical polypeptide)	Possible translation products		M_r of		
		M_r	stop codons	radioactive OmpA′ fragments observed after		stained OmpA′ fragments
				45 s	1 h	
pTU302						
pOMPA21	24500	37000	TAA^{335}			
		30000	TAG^{275}	33000		
			TAG^{277}	30000		
		29000	TAG^{268}	**29000**	**29000**	29000
		27000	TAG^{246}	28000		
					24000	**24000**
					22000	
pOMPA21-16	20500	27000	TAG^{248}	**27000**	**27000**	
			TAG^{250}			
		26000	TAG^{241}			
				25000	25000	
		24000	TAG^{219}		**24000**	**24000**
		22000	TGA^{200}	22000	22000	
pOMPA21-22	20500	DNA not sequenced		**29000**	29000	
				24000	**24000**	**24000**
					22000	
pOMPA21-11	14400	15000	TAA^{143}	16500		
				15000	none	none
pOMPA21-30	4900	8800	TAA^{81}			
		7300	TGA^{67}	none	none	none
		5500	TGA^{51}			

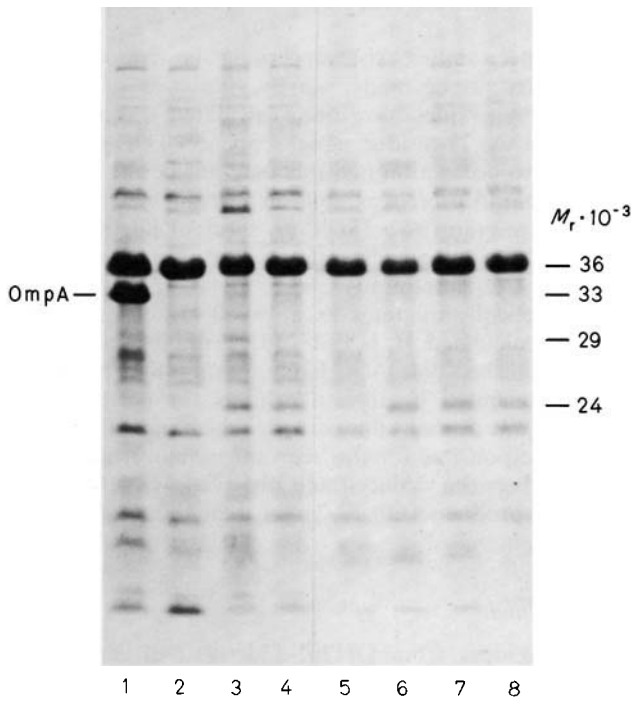

Fig. 4. *Stained sodium dodecylsulfate/polyacrylamide gel electrophoretograms of cell envelopes.* (1) Strain JC6650 (*ompA*$^+$); (2) strain UH201; (3–8) UH201-harboring plasmids pTU302, pOMPA21-2, pOMPA21-11, pOMPA21-16, pOMPA21-18, and pOMPA21-23, respectively. Note that as reported before [9] pTU302 generates a polypeptide of M_r 29000 in addition to the 24000-M_r fragment

envelope profiles from strains exhibiting phage resistance showed no OmpA′ products that were detectable by staining. The M_r of all the possible polypeptides which could be produced by the shortened *ompA′* genes have been determined (Table 4). From plasmids pOMPA21-16 proteins of M_r = 27000, 26000, 24000 and 22000 were expected; however, only one product, M_r = 24000, was found by staining. Thus the following questions arose: why were the proteins the same size in all cases where phage sensitivity was observed although the *ompA′* genes are of different sizes and why was no product from pOMPA21-11 found?

It was shown previously [9] that the major protein (M_r = 24000) produced by pTU302 is the proteolytically derived product of a larger polypeptide which has apparently lost residues from its CO_2H terminus. To examine whether a similar process was responsible for the 24000-M_r proteins produced by plasmids pOMPA21-16 and pOMPA21-22, the experiments were repeated using a radioactive labelling technique in which cells were pulse-labelled at 25 °C with [^{35}S]methionine for 45 s and the labelled products analysed by an immunoreplica assay. Part of the culture was processed immediately after the pulse and the remainder incubated for a further 60 min, thereby effecting an internal chase. The results of these experiments are shown in Fig. 5 and summarized in Table 4. It is apparent that the larger polypeptides, predicted by the DNA sequence data, were synthesized but were unstable. In the cases of pTU302, pOMPA21-16, and pOMPA21-22 the proteins appear to have been degraded to the 24000-M_r product and this occurred despite the differences in the residual *ompA′* sequences.

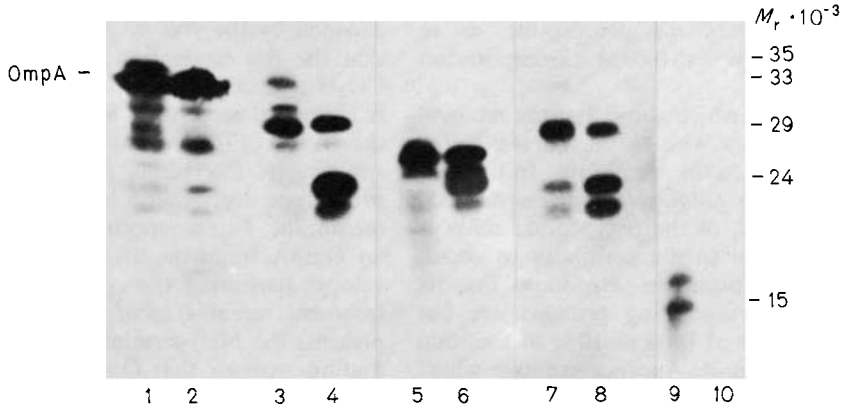

Fig. 5. *Fluorographs of immunoreplicas.* Cells were labelled with [^{35}S]methionine for 45 s and either processed immediately (lanes 1, 3, 5, 7, and 9) or incubated for a further 1 h (lanes 2, 4, 6, 8, and 10). Lanes 1, 2: strain JC6650 (*ompA*$^+$), strain UH201-3 with plasmid pTU302; lanes 5, 6: same strains with plasmid pOMPA21-16; lanes 7, 8: same strains with plasmid pOMPA21-22; lanes 9, 10: same strains with plasmid pOMPA21-11. The band above the OmpA protein in lane 1 is the pro-OmpA protein, and those below are also *ompA* products [42]. The band above the 15000-M_r polypeptide in lane 9 may well be its precursor

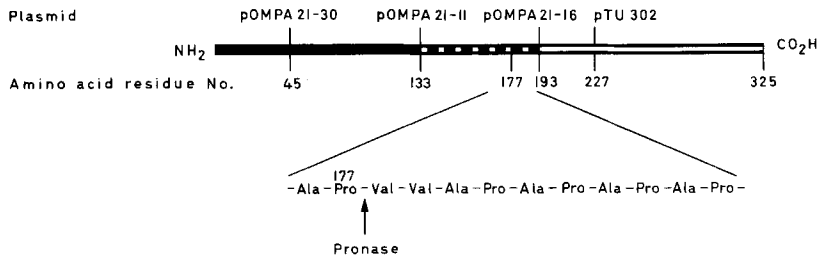

Fig. 6. *A model for the organisation of the OmpA protein.* The 325-residue OmpA protein is shown in three parts. The open region represents those parts which are not required for incorporation of the protein into the outer membrane. The filled-in region, residues 1–193, corresponds at least partly to the membrane domain of the protein. The NH_2-terminal portion represented by the solid line, residues 1–133, does not suffice for membrane incorporation but in combination with the hatched region, residues 134–193, membrane incorporation is achieved. All known functions of the OmpA protein are associated with the first 193 residues. The CO_2H-terminal moiety composed of residues 178-325 is exposed in the periplasm (unpublished data)

Proteins of the size predicted for pOMPA21-11 ($M_r \approx 15000$) were detected but these were considerably more unstable and appear to be completely degraded. No product from the *ompA'* gene of pOMPA21-30 was observed. However, as only 45 OmpA-specific residues remain, this short sequence may no longer exhibit sufficient antigenic sites to be recognized by the antiserum.

In view of the uncertainties concerning M_r determination for the OmpA protein and products derived from it, we cannot interpret the experimental values unambiguously. However, it is clear that proteins corresponding to the products of translation terminated by the major stop codons (TAA or more than one TAG codon) were observed. Furthermore, the TGA stop codon clearly constitutes an inefficient stop signal in this strain (Fig. 5 and Table 4).

DISCUSSION

Plasmid pTU302 carries the *ompA'* gene coding for amino acid residues 1–227 of the 325-residue OmpA protein. In this study we have reduced the size of the cloned chromosomal DNA in pTU302 thereby creating pOMPA21. It was then possible, essentially by using the single *Bam*HI site in plasmid pOMPA21 as an *ompA'*-specific target, to shorten the gene fragment further from its CO_2H terminus.

Requirements for the Incorporation of OmpA' Fragments into the Outer Membrane

The results show that at least 193 NH_2-terminal amino acid residues suffice for an apparently correct incorporation of OmpA' fragments into the outer membrane. However, 133 NH_2-terminal residues are insufficient for this purpose. These data should be considered together with the two-domain structure into which the protein may be subdivided structurally and most likely functionally (see Fig. 6). The evidence for this is as follows. Proteases, including pronase, when acting on cell envelopes remove the CO_2H-terminal parts of the OmpA protein [34]. Pronase cuts the protein at residue 177 [3] and the remaining 19000-M_r NH_2-terminal moiety retains phage receptor activity [34] and remains associated with the outer membrane. At the pronase cleavage site the conspicuous sequence -Ala^{176}-Pro-Val-Val-Ala-Pro-Ala-Pro-Ala-Pro-Ala-Pro^{187} exists [3] and the similarity of this sequence to such proline-rich sequences at the hinge regions of immunoglobulins has been pointed out [3,25]. It should also be noted that the pronase-resistant fragment is almost the same size as that coded for by the *ompA'* gene fragment present in pOMPA21-16. In conclusion, we can view the part of the OmpA protein encompassing residues 1 to about 180 as being the membrane protein proper. Thus, the loss of about 50, possibly less, CO_2H-terminal amino acid residues

from the membrane domain of the polypeptide, as in pOMPA21-11, suffices to interfere with its stable incorporation into the outer membrane.

Other examples are known which show that the removal of parts of a protein interferes with its membrane translocation or its incorporation into the membrane. In the case of the TEM β-lactamase from *Salmonella typhimurium* [35] it appears that most, if not all, of the polypeptide chain is required for sucessful secretion to the periplasm to occur. When chain-terminating mutations are introduced into its structural gene, *bla*, the corresponding proteins are not secreted even though they lack as little as 10% of the total residues from the CO_2H terminus. Another example where substantial parts of an outer membrane protein is required for membrane incorporation is that of the receptor for phage λ, the LamB protein [36–38].

Stable and Unstable Translation Products

We do not know whether any of the unstable translation products still possesses the signal sequence. Furthermore, except for the major 24000-M_r protein which is stable and found in the outer membrane, nothing is known about the cellular location of these peptides. Preliminary attempts to localize the pOMPA21-11 products have failed, probably because of their extreme instability. Since it is known that the first 177 residues of the OmpA protein are completely resistant to proteolytic attack due to their association with the membrane [3], it seems unlikely that the pOMPA21-11-encoded fragments are membrane-incorporated. But we cannot exclude the possibility that they reach the outer membrane and are degraded there, or that they are released into the medium. However, three possible explanations for the failure of membrane incorporation exist. Firstly, the whole membrane domain of the OmpA protein may have to assume a certain conformation in order to be transferred to the outer membrane. Secondly, a specific interaction with the outer membrane's lipopolysaccharide [34] may be required as was recently proposed by Beher et al. [39]. Thus, the lipopolysaccharide recognition site may be lacking in OmpA′ fragments such as those coded for by pOMPA-21-11. Furthermore, both recognition of lipopolysaccharide and correct folding may also be interfered with by the nine CO_2H-terminal amino acid residues encoded by the adaptor DNA (Fig. 3). Thirdly, the possible loss of OmpA fragments into the medium could indicate the absence of stop-transfer [40,41] or dissociation sequences [38]. This last possibility seems somewhat less likely because it would indicate that the polypeptide is inserted into the outer membrane during synthesis, and this is apparently not the case [42]. Whichever of these possibilities is correct remains to be elucidated. It is clear however, that most, if not all, of the membrane domain of the OmpA protein is required for correct incorporation into the outer membrane to occur.

The M_r of that part of the OmpA′ protein encoded by the *ompA′* gene in pTU302 is 24485 whereas the corresponding portion encoded by the shortened *ompA′* genes in pOMPA-21-22 is 20580 (Table 4). *In vivo*, all three plasmids direct the synthesis of larger proteins which possess CO_2H-terminal residues coded for by the vector DNA. However, in all three instances these proteins are subsequently degraded to stable, membrane-incorporated products of M_r 24000. In the case of pTU302 this product must consist almost entirely of *ompA′*-encoded residues while the products of the other two plasmids must still possess some 30–40 CO_2H-terminal residues, encoded by the vector, in addition. It is not only apparent that the cell recognizes and degrades part of the artificial CO_2H-terminal extensions but also clear that this degradation is limited in some way. Since none of the three proteins has the same CO_2H terminus, this process is not sequence-specific. It thus seems probable that the protection from proteolytic degradation is afforded the proteins by the outer membrane. This is supported by the fact that trypsin generates an OmpA fragment of M_r 24000 when acting on cell envelopes harboring the complete protein. Furthermore, this fragment remains incorporated in the membrane and represents the NH_2-terminal part of the protein [3,34]. These findings suggest that OmpA fragments, such as those produced by pOMPA21-16 and pOMPA21-22, could be used as export vehicles for small proteins of interest like insulin. In this case the relevant genes could be fused by genetic engineering techniques behind a part of the *ompA′* gene encoding sufficient of the protein's membrane domain to ensure membrane incorporation.

The findings of this and earlier studies [3,27,34] have been used to construct a functional model of the OmpA protein and this is presented in Fig. 6. It should be pointed out that those parts of the protein which are involved in colicin L-JF246 uptake and conjugation are situated within the first 193 residues. Likewise, the phage receptor site is located within the first 177 residues [34]. Finally, it should be noted that a function has yet to be discovered for the CO_2H-terminal part of the protein which contains some 140 residues, most, if not all, of which are located in the periplasm (unpublished observations).

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