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Gene fusions using the *ompA* gene coding for a major outer-membrane protein of *Escherichia coli* K12

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It has been shown previously that fragments of the *Escherichia coli* major outer membrane protein OmpA lacking CO_2H-terminal parts can be incorporated into this membrane *in vivo* [Bremer et al. (1982) *Eur. J. Biochem. 122*, 223–231]. The possibility that these fragments can be used, via gene fusions, as vehicles to transport other proteins to the outer membrane has been investigated.

To test whether fragments of a certain size were optimal for this purpose a set of plasmids was prepared encoding 160, 193, 228, 274, and 280 NH_2-terminal amino acids of the 325-residue OmpA protein. The 160-residue fragment was not assembled into the outer membrane whereas the others were all incorporated with equal efficiencies. Thus, if any kind of OmpA-associated stop transfer is required during export the corresponding signal might be present between residues 160 and 193 but not CO_2H-terminal to 193.

The *ompA* gene was fused to the gene (*tet*) specifying tetracycline resistance and the gene for the major antigen (*vp1*) of foot-and-mouth disease virus. In the former case a 584-residue chimeric protein is encoded consisting NH_2-terminally of 228 OmpA residues followed by 356 CO_2H-terminal residues of the 396-residue 'tetracycline resistance protein'. In the other case the same part of OmpA is followed by 250 CO_2H-terminal residues of the 213-residue Vp1 plus 107 residues partly derived from another viral protein and from the vector. Full expression of both hybrids proved to be lethal.

Lipophilic sequences bordered by basic residues, present in the non-OmpA parts of both hybrids were considered as candidates for the lethal effect. A plasmid was constructed which codes for 280 OmpA residues followed by a 31-residue tail containing the sequence: -Phe-Val-Ile-Met-Val-Ile-Ala-Val-Ser-Cys-Lys-. Expression of this hybrid gene was lethal but by changing the reading frame for the tail to encode another, 30-residue sequence the deleterious effect was abolished. It is possible that the sequence incriminated acts as a stop signal for transfer through the plasma membrane thereby jamming export sites for other proteins and causing lethality. If so, OmpA appears to cross the plasma membrane completely during export.

The OmpA protein is one of the abundant polypeptides of the *Escherichia coli* outer membrane (for a recent review see [1]). The trans-membrane protein [2] consists of 325 amino acid residues [3] and is synthesized as a precursor, the pro-OmpA protein, with a 21-residue signal sequence [4, 5]. The protein is arranged in the outer membrane such that an NH_2-terminal part, involving an area around residue 70, is exposed at the cell surface [6] and that a CO_2H-terminal moiety, most likely starting around residue 177, is largely located in the periplasm [3, 7].

In previous studies we prepared fragments of the cloned *ompA* gene and showed that OmpA fragments consisting of 193–228 NH_2-terminal residues are exported to the outer membrane and exhibit all known functions of the protein while fragments comprising 45 or 133 residues could not be detected in the membrane [8, 9]. For theoretical and practical reasons we wished to explore whether such exported fragments can be used, via gene fusions, as vehicles to transport other proteins to the outer membrane. This is of theoretical interest since it is largely unknown to what extent polypeptides, not designed for export, can be forced to follow, in such hybrids, the leading moiety along the export pathway. Furthermore, practical applications can be envisaged since such hybrid proteins containing part of OmpA, if incorporated into the outer membrane, would most likely not only be rather easy to isolate but also be produced in large quantities.

In this communication we describe the effects of expression of hybrid genes in which *ompA* segments of different lengths have been fused to nucleotide sequences specifying various polypeptides.

MATERIALS AND METHODS

Bacteria, the ompA *gene used, biological assays and nomenclature*

The strains used are listed in Table 1. A mutant *ompA* gene was employed, which carries a d(TAG) stop codon corresponding to amino acid residue 7 [4, 8]. Thus, in a suppressor-free background plasmids with potentially lethal *ompA* hybrids can be amplified. In the presence of a weak suppressor (strain UH100, the suppressor has not been identified and it may be of the ochre type) the expression of this allele is very low and the product cannot be detected on SDS/polyacrylamide gel elec-

Enzymes. T4 polynucleotide ligase (EC 6.5.1.1); DNA polymerase I (EC 2.7.7.7); S1 nuclease (EC 3.1.30.1); restriction endonucleases: *Bam*HI (EC 3.1.23.6); *Bgl*II (EC 3.1.23.10); *Eco*RI (EC 3.1.23.13); *Hin*dII (EC 3.1.23.20); *Hin*dIII (EC 3.1.23.21); *Hin*fI (EC 3.1.23.22); *Sal*I (EC 3.1.23.37); *Xma*I (EC 3.1.23.44); *Sph*I (EC 3.1.23.–).

Table 1. *Strains of* Escherichia coli *used*
Nomenclature is according to Bachmann and Low [44]

Strain	Origin	Genotype
UH100	W620 [8]	*thi pyrD gltA galK rpsL trp recA ompA*
UH201-3	UH201 [9]	*lac supF rpsL recA ompA*
$F^-Z^-\Delta M15$	U. Rüther [47]	*[lac pro]$^{\Delta}$ thi ara rpsL recA* ϕ*80 lac* Z^{Δ} M15
χ984	R. Curtiss [48]	*minA minB purE pdxC his rpsL ilv met cycA cycB xyl*

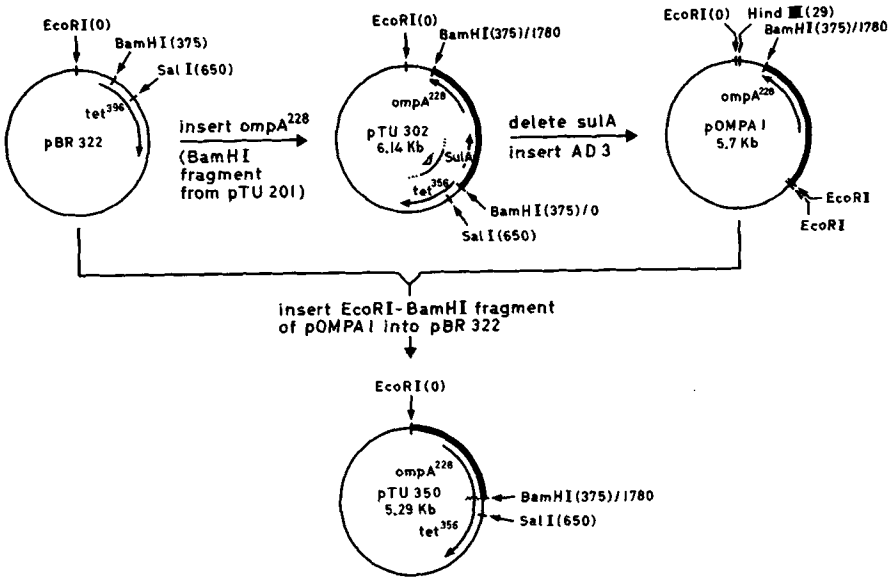

Fig. 1. *Construction of plasmids.* The thin parts of the circles represent pBR322 sequences with map positions [14] in brackets, the heavy segments represent *E. coli* DNA with map positions according to [4]. Δ in pTU302 indicates the extent of the deletion leading to pOMPA1, the wavy line in pTU350 the fusion joint between *ompA* and *tet*. Kb means $\times 10^3$ base pairs

trophoretograms. However, when UH100 harbors plasmids carrying this gene it becomes sensitive to a host range mutant (K3h1, [10]) of the OmpA-specific phage K3 while remaining resistant to K3. In the presence of the amber suppressor *supF* (strain UH201-3) wild-type amounts of OmpA are produced even if high-copy-number plasmids are used, since the suppressor tRNA becomes limiting. Consequently the problem of the lethal gene dosage effect, exerted by the wild-type *ompA* gene, can be overcome [8, 11]. Such strains are sensitive to phages K3 and K3h1.

The genes *ompA*, *lamB*, *vp1*, and *tet* code for proteins OmpA, the phage λ receptor LamB (physiologically a maltose-maltodextrin porin) [12, 13], the surface protein Vp1 of the foot-and-mouth disease virus, and Tet, a 396-residue protein [14a] involved in the expression of tetracycline resistance. Gene fragments, e. g. $ompA^{228}$, are given a superscript indicating the number of amino acid residues of the corresponding mature protein still encoded. The pOMPA plasmids were constructed in Heidelberg, the pTU plasmids in Tübingen. All experiments were performed at 37 °C.

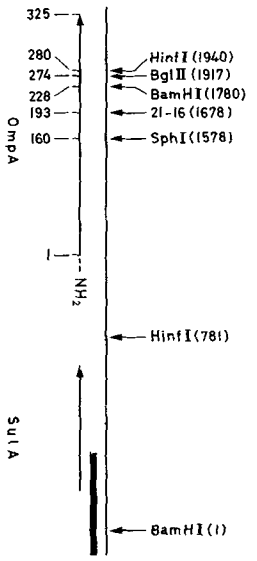

Fig. 2. *The* sulA-ompA *area of pTU201 [8, 45].* The bar above SulA indicates the approximate extent of the deletion in pOMPA1. The base-pair numbers in brackets are according to [4]. The numbers below OmpA show up to which amino acid residue of the mature protein the various fragments still code for. 21-16: end of *ompA* in pOMPA21-16 [9]. Stippled line on OmpA: signal sequence of the pro-OmpA protein

Construction of plasmids (Fig. 1 and 2)

pTU350. Plasmid pTU302 [8] was opened at the *Sal*I site, treated with exonucleases III and S1, and religated in the presence of synthetic *lac* operator fragment AD3 (containing two *Eco*RI sites) as described [9]. From the resulting plasmid

pOMPA1 the *Eco*RI-*Bam*HI fragment carrying $ompA^{228}$ was ligated into pBR322 [15], which had been cut with *Eco*RI and *Bam*HI.

pOMPA-FMDV. The *Bam*HI-*Hin*d III fragment from plasmid FMDV1034 [16, 17] carrying $vp1^{205}$ was ligated into pOMPA1, which had been cut with *Bam*H1 and *Hin*d III.

pTU400, 401, 402, and 403. For these plasmids the *Hin*fI-*Bam*HI (to yield pTU400 carrying $ompA^{228}$) the *Hin*fI-*Bgl*II (to yield pTU401 carrying $ompA^{274}$) and the *Hin*fI-*Hin*fI (to yield pTU402 carrying $ompA^{280}$) fragments indicated in Fig. 2 and isolated from plasmid pTU201 [8] were ligated into pUC8 [18]. For pTU400 and 401 the fragments to be transferred to pUC8, which was linearized with *Hin*dII, were provided with blunt ends by polishing with the Klenow fragment of DNA polymerase I. The same procedure was used for pTU402 except that pUC8 was opened with *Xma*I. The ligated plasmids were transformed into strain $F^-Z^-\Delta M15$. Cells harboring hybrid plasmids were detected as white colonies on L-broth agar containing ampicillin (20 μg/ml), isopropyl thiogalactoside (1 mM), and 5-bromo-4-chloroindolyl galactoside (40 μg/ml). pTU402 was linearized with *Eco*RI, the sticky ends were filled in as indicated above, and religation yielded pTU403.

pTU399. pOMPA21 [9] (a deletion produced *in vitro* and almost identical to pOMPA1 but not containing the AD3 linker) possesses the single *Sph*I site within *ompA* shown in Fig. 2. It was opened with *Hin*dIII and *Sph*I, treated with exonuclease III, and religated upon generation of blunt ends as described above.

In detail these experiments were performed as described previously [9] or following standard methods as given, e. g. in [19]. The enzymes were obtained from Bethesda Research Laboratories, Boehringer Mannheim, or New England Biolabs.

Other experimental procedures

In several cases the construction of the plasmids was verified by DNA sequencing. This was performed by the method of Maxam and Gilbert [20] when the vector was pBR322 and by the dideoxy chain-termination procedure [21] employing the reverse-sequencing primer (New England Biolabs), [22] when pUC8 was used.

Preparation and labeling of minicells from strain χ 984 was performed exactly as described [11]. Also, pulse labeling of cells growing at 25 °C, preparation of envelopes thereof, SDS/polyacrylamide gel electrophoresis and pertinent immunoreplicas were performed as described [9].

All plasmids except pOMPA-FMDV, and pTU403 were used to program protein synthesis *in vitro* according to Collins [23] with the modifications of Pratt et al. ([24], for details cf. [25]). Immunoreplicas of SDS/polyacrylamide gel electrophoretograms of such mixtures (Fig. 4), aside from the presence of the expected products (Table 2), invariably showed a strongly radioactive protein with $M_r \approx 30\,000$ (band X in Fig. 4) when plasmids pOMPA21-16 (Fig. 2), pTU400, 401, or 402 had been used; it was absent with plasmids carrying shorter *ompA* fragments (pTU399 and pOMPA21-11 [9]). We do not know which process in the system *in vitro* causes the appearance of this *ompA*-derived protein.

RESULTS

Considerations for the use of OmpA fragments as export vehicles

It has been shown that hybrids, consisting NH_2-terminally of the outer membrane porin LamB and CO_2H-terminally of most of the cytosolic β-galatosidase, can be incorporated into the outer membrane. Efficient export is only obtained when about 60% of LamB is present [26, 27] and full expression of this hybrid protein is partially tolerated by the host cells. When somewhat smaller parts of LamB are present greatly reduced amounts of the hybrid polypeptides are found in the outer membrane and full expression is lethal [12, 27]. The authors explained these effects by proposing the existence of a ribosome-dissociation sequence within LamB [26]: the membrane-bound ribosome synthesizing LamB is thought to dissociate from the membrane when this sequence appears. Thus, in those hybrids where the fusion joint was downstream from this sequence export can occur because the process of membrane incorporation would be finished before the part corresponding to the foreign polypeptide appeared. Fusions upstream of the dissociation sequence would be lethal if the foreign part of the hybrid, not designed for export, interferes with continued transfer through the membrane. Consequently the ribosome is unable to leave the membrane and jamming of the export sites for other proteins occurs.

Clearly, if this model applies to the export of OmpA and if OmpA is to be utilized as an export vehicle, for proteins not normally exported we ought to know where its ribosome dissociation sequence is located. One would expect that OmpA fragments not possessing this sequence, in contrast to those harboring it, would be incorporated rather inefficiently, or possibly not at all, into the outer membrane. We have, therefore, prepared a new set of plasmids bearing different lengths of the *ompA* gene allowing us to determine the efficiency of export of the corresponding OmpA fragments.

Efficiency of export of protein fragments

As detailed in Materials and Methods all experiments were performed with derivatives of an *ompA* gene, which carries a d(TAG) stop codon. In the presence of the amber suppressor *supF* wild-type amounts of this OmpA protein are produced even when high-copy-number plasmids are used, because the suppressor tRNA becomes the limiting factor.

Plasmids pTU399, pOmpA21-16, pTU400, and pTU401 carry hybrid genes in which 160, 193, 228, and 274 codons of *ompA* are fused to short reading frames in the vectors (Fig. 3). Expression of these genes in strain UH201-3 caused sensitivity to phage K3 except in the case of pTU399, where the cells remained resistant to phages K3 and K3h1. No OmpA-derived protein could be detected by electrophoretic analysis of envelopes from cells carrying pTU399; representative outer membrane protein profiles from strains harboring the other plasmids are shown in Fig. 4. In Table 2 the sizes of the products, calculated from the DNA sequences, are compared with those synthesized *in vitro* (Fig. 5) and *in vivo*. (Plasmids pTU402 and 403 shown in this table will be taken up in the last section.)

Firstly, the data show that the OmpA fragment, comprising 160 residues, encoded by pTU399, cannot be stably incorporated into the outer membrane. Secondly, and as described in more detail previously [9], in all other cases degradation of the OmpA hybrids occurred *in vivo* since a product of $M_r \approx 24\,000$ was always found. In the case of pTU401, where most of the product consists of OmpA sequence, partial resistance to this proteolysis exists as demonstrated by the presence of an additional species of $M_r \approx 31\,000$. Thirdly, and as judged from a number of similar electrophoretograms, there was no significant difference in the amounts of the OmpA fragments present in envelopes from cells harboring the various plasmids; we

```
          —ompA——  ————————————AD3————————————————————————
             1678
pOMPA21-16  AAG CAC GAT CCG GAA TTC AAA TTG TGA GCG GAT AAC AAT TTG
             193                         200
            Lys His Asp Pro Glu Phe Lys Leu Stp Ala Asp Asn Asn Leu
            ——————————— ——————pBR322————————————————————————————>
                         (353)
            AAT TCC GGA TCT GAT CGC GTA GTC GAT AGT GGC TCC AAG TAG
                                                            219
            Asn Ser Gly Ser Asp Arg Val Val Asp Ser Gly Ser Lys Stp
            CGA AGC GAG CAG GAC TGG GCG GCG GCC AAA GCG GTC GGA CAG
            Arg Ser Glu Gln Asp Trp Ala Ala Ala Lys Ala Val Gly Gln
            TGC TCC GAG AAC GGG TGC GCA TAG AAA TTG CAT CAA CGC ATA
                                        241
            Cys Ser Glu Asn Gly Cys Ala Stp Lys Leu His Gln Arg Ile
            TAG CGC TAG
            248     250
            Stp Arg Stp
```

```
            —ompA— ———pBR322————————————————————————————————————>
                 1578(31)
pTU399      AAC GGC TTA TCG ATG ATA AGC TGT CAA ACA TGA GAA TTC TTG
                 160                             169
            Asn Gly Leu Ser Met Ile Ser Cys Gln Thr Stp Glu Phe Leu
            AAG ACG AAA GGG CCT CGT GAT ACG CCT ATT TTT ATA GGT TAA
            Lys Thr Lys Gly Pro Arg Asp Thr Pro Ile Phe Ile Gly Stp
```

```
            —ompA——— ————pUC8————>
                 1783
pTU400      TTG GAT CGA CGG ATC CCC GGG AAT TCG TAA
                228
            Leu Asp Arg Arg Ile Pro Gly Asn Ser Stp
```

```
           —ompA—— ————pUC8————>
               1921
pTU401     AAG ATC GAC GGA TCC CCG GGA ATT CGT AAT CAT GGT CAT AGC
               274
           Lys Ile Asp Glu Ser Pro Gly Ile Arg Asn His Gly His Ser
           TGT TTC CTG TGT GAA ATT GTT ATC CGC TCA CAA TTC CAC ACA
           Cys Phe Leu Cys Glu Ile Val Ile Arg Ser Gln Phe His Thr
           ACA TAC GAG CCG GAA GCA TAA
           Thr Tyr Glu Pro Glu Ala Stp
```

```
            —ompA—— ————pUC8————>
                1942
pTU402      GGC GAA TGG GAA TTC GTA ATC ATG GTC ATA GCT GTT TCC TGT
                280
            Gly Glu Trp Glu Phe Val Ile Met Val Ile Ala Val Ser Cys
            GTG AAA TTG TTA TCC GCT CAC AAT TCC ACA CAA CAT ACG AGC
            Val Lys Leu Leu Ser Pro His Asn Ser Thr Gln His Thr Ser
            CGG AAG CAT AAA GTG TAA
            Arg Lys His Lys Val Stp
```

```
            —ompA—— ————pUC8————>
                1942
pTU403      GGC GAA TGG GAA TTA ATT CGT AAT CAT GGT CAT AGC TGT TTC
                280
            Gly Glu Trp Glu Leu Ile Arg Asn His Gly His Ser Cys Phe
            CTG TGT GAA ATT GTT ATC CGC TCA CAA TTC CAC ACA ACA TAC
            Leu Cys Glu Ile Val Ile Arg Ser Gln Phe His Thr Thr Tyr
            GAG CCG GAA GCA TAA
            Glu Pro Glu Ala Stp
```

Fig. 3. *CO_2H-terminal sequences of OmpA hybrids.* The data for pOMPA21-16 are from [9]. Strain UH201-3 carrying this plasmid has been subjected to a [^{35}S]methionine pulse and a major OmpA-specific product of $M_r \approx 27000$ was found [9], which corresponds to a protein of about 245 residues, i. e. the main translation termination occurred around the stop codons (Stp) at positions 241, 248, and 250. *supF* in UH201-3 causes incorporation of tyrosine which will be present at positions 200 and 219. The d(TGA) stop codon in position 169 of pTU399 is very inefficient in strain UH201-3 [9], the amino acid incorporated is not known. The orientation of all fragments in pUC8 was found to be the same, the *ompA* reading frame continues upstream into the non-coding strand of the adjacent part of the *lac* operon [18, 46]. The amino acid sequence proposed to cause lethality is underlined in pTU402. Underlined nucleotides: polished *Bam*HI, *Bgl*II, and *Hin*fI sites in *ompA* of pTU400, 401, and 402 respectively. The broken line in pTU402 shows the *Eco*RI site, which was opened, filled in, and religated (underlined in pTU403). Arrows: *Hin*dII, *Hin*dII, and *Xma*I sites in the pUC8 parts of pTU400, 401, and 402, respectively. Nucleotide coordinates are given as in Fig. 2, coordinates have not been assigned to pUC8. Hyphers representing both phosphodiester and peptide links have been omitted

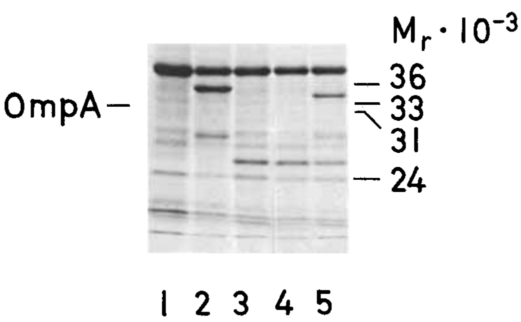

Fig. 4. *Stained SDS/polyacrylamide gel electrophoresis of cell envelopes.* 1, strain UH201-3: 2, *ompA*$^+$ parent of UH201-3; 3, UH201-3 carrying pOMPA21-16 (*ompA*^{193}); 4, same strain carrying pTU400 (*ompA*^{228}); 5, same strain carrying pTU 401 (*ompA*^{274}). The products of the *ompA*^{193} and *ompA*^{228} genes migrated according to $M_r \approx 24000$; in addition to such a protein one with $M_r \approx 31000$ is present when *ompA*^{274} was used

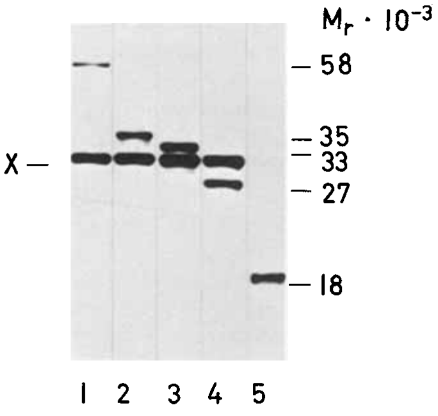

Fig. 5. *Fluorographs of immunoreplicas.* Proteins reacting with anti-OmpA serum synthesized *in vitro* using the plasmids: 1, pTU350 (*ompA-tet* fusion); 2, pTU100 (possessing the complete *ompA* gene [8]); 3, pTU401 (*ompA*^{274}); 4, pTU400 (*ompA*^{228}); 5, pTU399 (*ompA*^{160}). For band X see Materials and Methods

Table 2. *Products of partially deleted* ompA *genes*
The values in lanes 1 and 2 are approximations arrived at by using an average M_r of 110 per residue. These do not take into account the 21-residue signal sequence (M_r 1936) although this sequence is not removed from the products synthesized *in vitro*. Pro-OmpA (M_r 37000) and OmpA (M_r 35000) migrate electrophoretically as M_r 35000 and 33000 species, respectively [11], assuming the same anomalous behavior for the fragments good agreement is obtained between expected M_r (i. e. 2000 Da smaller than calculated from the DNA sequence) and those found for the products *in vitro*

Plasmid	M_r of OmpA-specific sequences still encoded	M_r of hybrid polypeptides		
		expected	found	
			in vitro	*in vivo*
pTU399 (*ompA*^{160})	17600	18500 or 20300[a]	18000	none
pOMPA21-16 (*ompA*^{193})	20500	27000	no tested[b]	24000
pTU400 (*ompA*^{228})	25000	25800	25000	24000
pTU401 (*ompA*^{274})	30100	33600	33000	31000+24000
pTU402 (*ompA*^{280})	30800	34200	34000	–[c]
pTU403 (*ompA*^{280})	30800	34100	not tested	31000+24000

[a] It is not known if the stop codon in position 169 (Fig. 3) is efficient in the system *in vitro*.
[b] For the product of this *ompA* fragment see legend to Fig. 3.
[c] Full expression *in vivo* is lethal (see text) and the product could, therefore, not be identified.

estimate that in each case the concentration of fragment(s) is about one-half of that obtained when a complete *ompA* gene is expressed (Fig. 4).

It is concluded that the sequence between residues 160 and 193 must be present for efficient incorporation of OmpA fragments into the outer membrane, and this area may contain a ribosomal dissociation sequence.

Such a sequence most likely does not exist between residues 193 and 274. Thus, if a hybrid gene were produced containing sufficient information to code for more than 193 NH_2-terminal OmpA residues fused to a second coding sequence, export of the hybrid protein may occur although there would be some danger of degradation.

Fusion of ompA *with the gene specifying tetracycline resistance and with the gene for the major antigen of foot-and-mouth disease virus*

To test the usefulness of the OmpA protein as an export vehicle we chose the *tet* and the *vp1* gene of the foot-and-mouth disease virus because the corresponding plasmids were easy to construct (see Materials and Methods and Fig. 1). In pTU350 the fused genes code for a chimeric protein containing 584 residues, of which 227 are from the NH_2 terminus of OmpA and 356 from the CO_2H terminus of the 396-residue Tet protein. The 228th residue (fusion codon) is common to both proteins. When used to program protein synthesis *in vitro*, pTU350 directed the production of $M_r \approx 58000$ polypeptide (Fig. 3), in fair agreement with the $M_r \approx 64000$ calculated for the hybrid polypeptide (the 10% difference may be attributable to the aberrant electrophoretic mobility of OmpA, see legend to Table 2). In pOMPA-FMDV the fused genes code for a hybrid containing 540 residues, of which 227 are again from the NH_2 terminus of OmpA. The 205-amino-acid-residue stretch from residues 228 to 433 corresponds to the mature Vp1 protein. Because the viral proteins are synthesized as a common polypeptide precursor the reading frame also codes for 77 residues of another viral protein and continues for a further 30 codons into the vector DNA before ending with a d(TAA) stop codon.

On introduction into strain UH100, pTU350 conferred K3h1 sensitivity although, as expected, too little protein was present to be detected by SDS/polyacrylamide gel electrophoresis. To obtain full expression of the hybrid the plasmid was placed into strain UH201-3 but the transformants, arising at normal frequencies, grew extremely slowly. It was, therefore, not possible to grow enough cells for the preparation of envelopes, as such cultures were always overgrown by faster growing derivatives. Ten of these were isolated and analysed in detail. In three cases K3 sensitivity was observed and this was accompanied by the presence of a $M_r \approx 24000$ polypeptide in cell envelopes in quantities as shown for, e. g. pTU400 in Fig. 4. The remaining seven isolates produced no detectable OmpA-derived protein. These results show that there is a strong selective pressure for mutants which either do not produce the hybrid protein (or cannot export it) or are unable to express the part derived from the *tet* gene. Obviously, full expression of the *ompA-tet* fusion gene is semilethal.

Plasmid pOMPA-FMDV in strain UH100 also conferred sensitivity to K3h1. However, transformation into strain UH201-3 yielded clones at frequencies of about $10^{-4}\times$ those observed with UH100. Ten such clones were recovered and all proved to be resistant to phages K3 or K3h1. Also, no trace of the hybrid or a fragment derived there from could be detected in cell envelopes. Thus, full expression of this hybrid gene is lethal and in strain UH201-3 only mutants no longer synthesizing or exporting the product could be found.

Products of the ompA-tet *and* ompA-vp1 *genes* in vivo

When synthesized at low levels the products of the fused genes confer K3h1 sensitivity to the host, proving that at least their OmpA parts are in the outer membrane. This raised the question of why the hybrid proteins were lethal when synthesized in large quantities.

As shown previously [9] and demonstrated again here the primary translation products of $M_r \approx 27000$ and $M_r \approx 33000$, respectively, encoded by the smaller hybrid genes present in pOMPA21-16 or pTU401 are completely or partially degraded at their CO_2H termini so that the final product in the cell envelope is of $M_r \approx 24000$. One reason for the lethality, observed with the OmpA-Tet and OmpA-Vp1 hybrids, could be that the cell starts to export them but that their non-OmpA parts cannot follow the route to the outer membrane, become

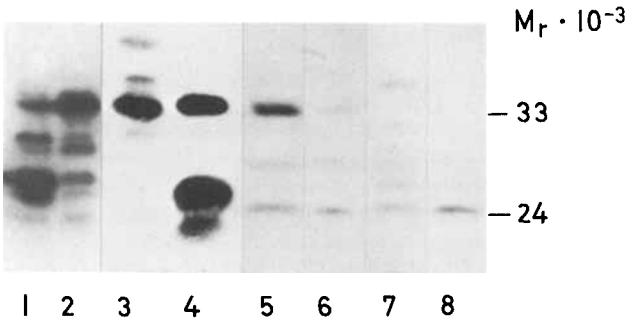

Fig. 6. *Fluorographs of immunoreplicas.* Minicells harboring pTU350 (*ompA-tet* fusion, lane 1) or pTU201 (complete *ompA* gene, lane 2) had been incubated for 1 h at 37 °C in the presence of [^{35}S]methionine before processing for SDS/polyacrylamide gel electrophoresis and immunoreplica. Note that under such conditions small amounts of OmpA ($M_r \approx 33000$) were degraded. No product of the hybrid gene larger than 33 kDa was detectable. 3–8, cells growing at 25 °C and after labeling with [^{35}S]methionine for 15 s (lanes 3, 5, 7) followed by a chase with non-radioactive methionine for 1 h (lanes 4, 6, 8). 3, 4, strain UH201-3 harboring pTU302 (*ompA*^{228}); 5, 6, strain UH100 with the same plasmid; 7, 8, strain UH100 carrying pOMPA-FMDV. Lanes 3–6 are shown to demonstrate the large difference in the efficiency of the amber suppressor *supF* in UH201-3 and the unidentified weak suppressor in UH100, the film exposure time for 3 and 4 was 2 days, that for 5–8, 3 weeks. (For unknown reasons in UH100, but not in UH201-3, radioactivity in pulse-labeled OmpA fragments could be chased only partially into the final product)

stuck in the cytoplasmic membrane and block export sites for other proteins. If only small amounts of the hybrids are produced proteolysis of the type mentioned may remove their non-OmpA parts but this degradation may be too slow to remove these parts when large quantities of hybrids are synthesized. Increased jamming of export sites would then be lethal.

Some support for this view was provided by the following experiments. In one, plasmid pTU350 was transferred into the minicell-producing strain χ 984, which carries a suppressor of the same low efficiency as that in strain UH100. Minicells were incubated for 1 h in the presence of [^{35}S]methionine and the radioactively labeled proteins separated electrophoretically on an SDS/polyacrylamide gel, which was then subjected to immunoreplica. The resulting pattern is shown in Fig. 6. No trace of the complete hybrid protein (expected $M_r = 58000$) can be seen and the major product exhibits a mobility corresponding to $M_r \approx 24000$. In another experiment strain UH100, harboring plasmid pOMPA-FMDV, was grown at 25 °C, pulsed with [^{35}S]methionine, and labeled proteins reacting with anti-OmpA serum were identified in the same way (Fig. 6). Even after a 15-s pulse no trace of the complete hybrid protein (expected $M_r = 60000$) was found, the largest fragment migrates according to a $M_r \approx 33000$ and, after a chasing, the main remaining product exhibits a molecular weight of approximately 24000. It would, therefore, appear that degradation of the hybrid is already occurring during its synthesis.

Lethality of an OmpA fragment containing a signal-like sequence at its CO_2H terminus

If our interpretation is correct, namely, that the non-OmpA parts of the hybrids just described block the export machinery thereby jamming export sites for other proteins, this blockage may be caused by particular amino acid sequences [28, 29] or by these parts assuming conformations incompatible with export. There may also be other reasons for the deleterious effects observed and it would, of course, be very difficult to elucidate exactly which property of the hybrids causes lethality.

It has been found that expression of the vesicular stomatitis virus glycoprotein gene in *Escherichia coli* is lethal when the signal sequence (Lys-Lys-Leu-Leu-Thr-Leu-Ala-Phe-Leu-Phe-Ile-) of the glycoprotein is present and if it is preceeded NH_2-terminally by 10 other amino acid residues encoded by the expression vector [30]. Within the Tet fragment encoded on pTU350 a sequence exists to some degree resembling a signal sequence: -Arg^{129}-Ala-Arg-His-Phe-Gly-Leu-Met-Ser-Ala-Cys-Phe-Gly-Val-Gly-Met-Val-Ala-Gly-Pro-Val-Ala-Gly-Gly-Leu-Leu-Gly-Ala-Ile- [14a]. Vp1 does not contain a similar sequence; however, a stretch occurs consisting of predominantly hydrophobic residues bordered and interrupted by basic ones: -Ala^{106}-Tyr-His-Lys-Ala-Pro-Leu-Thr-Arg-Leu-Ala-Leu-Pro-Tyr-Thr-Ala-Pro-His-Arg-Val-Leu-Ala- [17]. Considering such sequences as candidates for inhibition of export we have looked for a simpler OmpA hybrid, which would possess, CO_2H-terminally, only a short non-OmpA part containing a signal-like sequence. It turned out that the construction of pTU402 would tail OmpA at residue 280 with 31 residues containing such a sequence (Fig. 3). Transformation into strain UH201-3 repeatedly yielded clones at frequencies of only about $10^{-4}\times$ those observed with UH100. Five such clones were recovered and proved to be resistant to K3h1. Cell envelopes of two of these were examined electrophoretically and found to be devoid of the expected fragment, i.e. a small fraction of the plasmids used had undergone mutational alterations preventing expression of the fusion gene or export of its product. Clearly, full expression of this gene fusion is lethal, indicating that the signal-like sequence present in the non-OmpA part of the hybrid may indeed cause lethality. This interpretation was corroborated by constructing pTU403 (Fig. 3), in which the 'toxic tail' is replaced by a different sequence of 30 residues. This plasmid could readily be introduced into strain UH201-3 and the transformants were sensitive to phage K3. Electrophoretic analysis of their cell envelopes revealed exactly the same profile as displayed for pTU401 in Fig. 5, i.e. two polypeptides with $M_r \approx 31000$ and $M_r \approx 24000$ were present at about equal quantities.

DISCUSSION

Although our attempts to use the OmpA protein as an export vehicle were unsuccessful the present study has provided valuable information about the mechanism of protein export. In this respect it should be of use to other workers interested in creating gene fusions with the aim of exporting hybrid proteins of interest.

Export of OmpA fragments

A comparison of the amounts of OmpA fragments present in the outer membrane showed no significant differences between fragments containing 193, 228, 274 or 280 OmpA residues. All were present at concentrations corresponding to about 50% of that of the complete OmpA protein. It should be pointed out that the discrepancy between the expression of the complete *ompA* gene and its shortened derivatives is not due to limited suppression of the amber mutation found in the latter, since wild-type amounts of protein are produced when a

complete *ompA* gene, with amber mutation, is present on a multicopy plasmid in this background (UH201-3 [8]). We believe that the lower concentration of the exported OmpA fragments may be due to decreased stability of the corresponding mRNAs since these all lack the transcriptional terminator [4, 5]. This is supported by the findings presented in [25] and by the observation that the *ompA* mRNA decays with a 3′ to 5′ directionality [31].

As described previously [9], the OmpA protein exhibits a two-domain structure with the NH_2-terminal part representing the membrane protein proper and the stretch from residue 177 to 325 being largely periplasmic. With regard to membrane incorporation the data presented in this communication define the boundary between these domains more precisely, as the removal of 33 amino acids from the CO_2H terminus of a 193-residue OmpA fragment suffices to completely prevent assembly into the outer membrane. Whether the part of the protein between residues 160 and 193 corresponds to the ribosome dissociation sequence or whether this region is required for the correct membrane conformation to be adopted, remains to be elucidated. It is, however, clear that none of the protein after position 193 is obligatorily required for membrane incorporation to occur although this region enables the protein to adopt a protease-resistant conformation. This is indicated by the ubiquitous occurrence of the $M_r \approx 24000$ species in all cases where the *ompA* gene has been shortened. It is interesting to note that where larger parts of OmpA are present the fragments exhibit increased stability as shown by the presence of larger products ($M_r \approx 31000$) encoded by pTU401 and pTU403. The OmpA protein contains two cysteine residues [3] near its CO_2H terminus and it is possible that these are involved in the formation of a disulfide bond, which is required for the protein to fold into a protease-resistant form. Unpublished studies from this laboratory show that if such a disulfide bond is present it is most likely of the intermolecular type.

A comparison of the export of OmpA with that of other truncated outer membrane proteins suggests that successful export cannot be generally correlated with the length of the polypeptide fragment but is instead specific for a given protein. Particularly revealing is the behavior of *Escherichia coli* porin fragments. With the maltodextrin porin, LamB, NH_2-terminal fragments fused to most of the cytosolic β-galactosidase can be very efficiently exported if the LamB part reaches a certain size (≈60% of the protein [26, 27]). In contrast, no fragments of two other porins, the PhoE and OmpF proteins, could be detected in the outer membrane. In the case of the 330-residue PhoE protein removal of only 50 CO_2H-terminal amino acids prevents membrane incorporation [32]. Likewise, although transposon insertions have been obtained throughout the ompF gene [33], the corresponding truncated OmpF proteins could not be detected. These three polypeptides serve very similar functions but while OmpF and PhoE are strikingly homologous in their primary structures no homology could be found between LamB and OmpF or PhoE [32, 34]. Taken together these findings suggest that different proteins probably have to assume unique conformations to be incorporated into the membrane and/or that the specific signals required for the export process do not have similar locations within the polypeptides.

Lethal export of hybrids

Export of the *ompA-vp1* and *ompA-tet* products is lethal when they are produced at high rates. If synthesized at low levels the complete hybrid proteins do not reach the outer membrane, the foreign parts are proteolytically removed and the OmpA fragment alone appears in the membrane. The proteolysis apparently occurs during synthesis of the polypeptides. These observations, together with the fact that the mere intracellular presence of Vp1 is not lethal [16], strongly suggest that it is the attempted export of the hybrids which causes cell death. As discussed already, we cannot pinpoint exactly why export of the hybrid proteins is blocked although it seems most likely that they get stuck in the plasma membrane and jam the export machinery as has been described for some MalE− or LamB−β-galactosidase hybrids [26, 27, 35].

The situation is somewhat clearer with the five hybrids shown in Fig. 3 and with pOMPA21 described earlier [9], i. e. concerning all exported OmpA fragments. In only one case (pTU402) did the foreign part of the hybrid protein lead to cell death. The 31-residue 'tail' attached to the 280th residue of OmpA contains a hydrophobic sequence, ending with a lysine residue (Fig. 3), which thus resembles a signal sequence. When present internally, similar sequences are known or are strongly suspected to anchor certain trans-membrane proteins in the membrane. Good examples are phage M13 coat protein [36], glycophorin [37, 38], hemagglutinin of influenza virus [39], membrane-bound immunoglobulin IgM [40], glycoprotein of the vesicular stomatitis virus [41] and the *E. coli* serine chemoreceptor [42]. Thus, when considered together with the vesicular stomatitis virus glycoprotein mentioned in Results, there is little doubt that it is the pseudo-signal sequence attached CO_2H-terminally which causes lethality in spite of the presence of 86% of the mature OmpA protein. As we have found no evidence for a ribosome-dissociation sequence being present between residues 193 and 280 it may be that during export OmpA completely crosses the cytoplasmic membrane and even exists in a non-membrane-associated state. In fact, it has been shown that OmpA is transiently present in the periplasmic space, on its route to the outer membrane, albeit under non-physiological conditions [43]. It seems, therefore, that the model for protein translocation involving a ribosomal dissociation sequence is not applicable in its entirety to the OmpA protein.

Studies are in progress designed to elucidate exactly why full expression of the hybrid gene present on pTU402 is lethal.

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