The EMBO Journal vol.9 no.6 pp.1991 – 1999, 1990

Extracellular transport of cholera toxin B subunit using *Neisseria* IgA protease β-domain: conformation-dependent outer membrane translocation

Thomas Klauser, Johannes Pohlner and Thomas F.Meyer

Max-Planck-Institut für Biologie, Abteilung Infektionsbiologie, Spemannstrasse 34, D-7400 Tübingen, FRG

Communicated by Ulf Henning

The β-domain of the *Neisseria* IgA protease precursor (Iga) provides the essential transport function for the protease across the outer membrane. To investigate the secretion function of the β-domain (Iga_β), we engineered hybrid proteins between Iga_β and the non-toxic 12 kd cholera toxin B subunit (CtxB) and examined their targeting behaviour in *Salmonella typhimurium*. We show that CtxB–Iga_β hybrid proteins integrate into the outer membrane, leading to the exposition of the CtxB moiety on the cell surface. Exposed CtxB can be degraded by externally added proteases like trypsin, but can also be specifically cleaved off from membrane-associated Iga_β by purified IgA protease. We further demonstrate that folding of the CtxB moiety at the periplasmic side of the outer membrane interferes with its translocation. Prevention of disulphide-induced folding in periplasmic CtxB renders the protein moiety competent for outer membrane transport. Iga_β may be of general interest as an export vehicle for even larger proteins from Gram-negative bacteria.

Key words: outer membrane targeting/protein export/secretion/surface exposition

Introduction

The *iga* genes of pathogenic *Neisseria* species code for extracellular proteins that cleave human IgA1 antibody. In contrast to most other exported proteins of Gram-negative bacteria, the *iga* gene alone is sufficient to direct selective extracellular secretion of IgA protease not only in *Neisseria* species, but also in *Escherichia coli* and in *Salmonella* species (Meyer *et al.*, 1987; Pohlner *et al.*, 1987a).

The mechanism of IgA protease secretion has been studied extensively using the *iga* gene product of *N.gonorrhoeae* strain MS11. The mature IgA protease (106 kd) is processed in several steps from a large precursor of 169 kd by signal peptidase and autoproteolytic cleavage (Halter *et al.*, 1984; Pohlner *et al.*, 1987b). The precursor consists of four structurally and functionally distinguishable domains: (i) an amino-terminal signal peptide which is assumed to participate in inner membrane transport, (ii) the actual protease domain, (iii) the α-domain, an extremely basic α-helical region which is secreted in conjunction with the protease and (iv) the β-domain (Iga_β) which harbours the essential functions for outer membrane transport. The carboxy-terminal Iga_β integrates into the outer membrane and mediates the translocation of protease domain and α-domain. These domains are then released from the cell by autoproteolytic cleavage as a single 121 kd protein (P121), which is subsequently converted into the 12 kd α-protein and a 109 kd protease intermediate (P109). P109 is finally processed to the mature IgA protease (106 kd) by a further autoproteolytic step. This model was recently confirmed for a number of variant IgA proteases from various *N.gonorrhoeae* strains, in which the α- and β-proteins differed substantially in size and sequence (Halter *et al.*, 1989). However, the core regions of the β-proteins are highly conserved and hence may contain the functions essential for the association of the β-domain with the outer membrane (unpublished). Deletions at the 3′ terminus of the iga_β region abolish protease secretion (Pohlner *et al.*, 1987b), while the α-domain, which was formerly believed to be part of the outer membrane transport machinery, can be deleted from the precursor without affecting extracellular secretion of IgA protease (Pohlner *et al.*, 1990). According to these results, Iga_β alone accomplishes the process of outer membrane transport of covalently attached proteins without requiring additional specific secretion functions.

These data prompted us to examine the idea of using the IgA protease secretion pathway to export foreign polypeptides from Gram-negative bacteria (Meyer *et al.*, 1987). One possibility of manipulating the *iga* gene product was to substitute completely the IgA protease domain and α-domain. This approach, which would eliminate IgA protease activity, was expected to result in the exposition of the passenger protein on the cell surface of the Gram-negative host bacteria, without autoproteolytic release of the protein into the medium.

For this purpose we chose the B subunit of cholera toxin (Lockman and Kaper, 1983). Cholera toxin (Ctx) is a multisubunit protein consisting of one A subunit and five identical B subunits (Ribi *et al.*, 1988). While the A subunit elicits toxicity in *Vibrio cholerae* infections, the non-toxic B subunits mediate binding to GM1 ganglioside, the toxin's receptor on eukaryotic target cells (Holmgren, 1981). The A and B subunits, which possess amino-terminal signal peptides (Mekalanos *et al.*, 1983), are independently transported across the cytoplasmic membrane (Hirst and Holmgren, 1987a). Subunit assembly is accomplished in the periplasmic space, where the holotoxin transiently accumulates (Hirst and Holmgren, 1987b). Intramolecular disulphide bond formation within each B subunit is a prerequisite for subunit assembly *in vivo* and *in vitro* (Hardy *et al.*, 1988). The holotoxin is released into the medium of *V.cholerae* by a yet unknown mechanism (Hirst and Holmgren, 1987a). In *E.coli*, however, the cholera toxin (like its *E.coli* counterpart Etx) is retained in the periplasm and not released into the medium (Pearson and Mekalanos, 1982; Mekalanos *et al.*, 1983), probably because this species is devoid of specific secretion functions for transport of Ctx through the outer membrane (Hirst and Holmgren, 1987a).

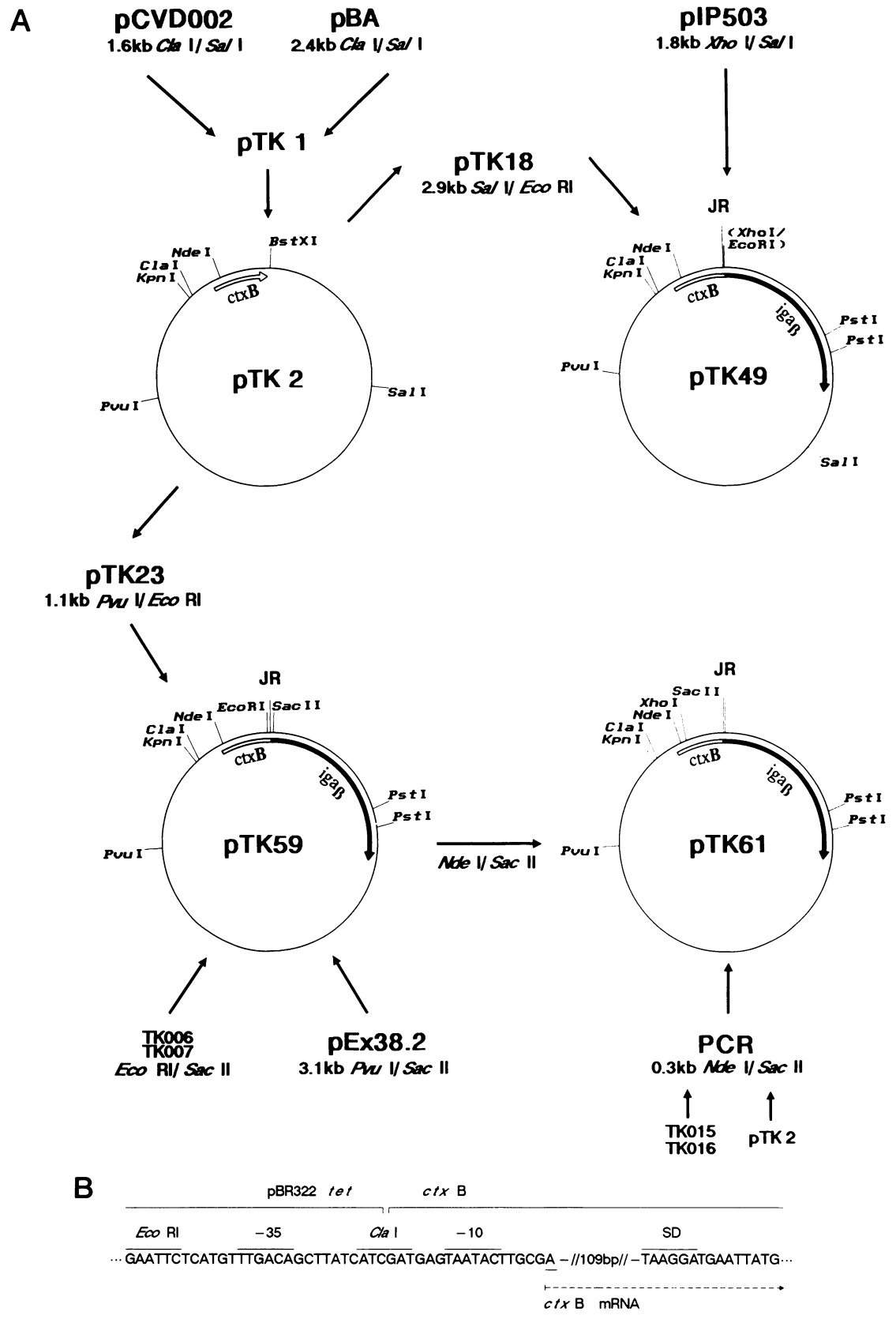

Fig. 1. (**A**) Derivation of plasmids. The main plasmids used in this study, carrying either the *ctx*B gene (pTK2) or *ctx*B–*iga*$_\beta$ gene fusions (pTK49, pTK59 and pTK61) are presented as circular maps. The joining region of the fusion partners are assigned 'JR', and the DNA fragments of parental plasmids used for cloning are indicated by name. The DNA fragment encoding mutagenized CtxB, which was made by polymerase chain reaction, is indicated by 'PCR'. TK006, TK007, TK015 and TK016 are the oligonucleotides used for cloning or PCR (see Table I). For detailed explanation see Materials and methods. (**B**) Structure of the constitutive expression signal P_k. The putative promotor units −35 and −10 of promotor P_k, the Shine–Dalgarno sequence (SD) and the positions for restriction sites are indicated. The transcriptional start, as detected by primer extension, is underlined. The *Eco*RI site of the promotor region was exchanged by 10mer *Kpn*I linkers in plasmids containing *ctx*B–*iga*$_\beta$ hybrid genes.

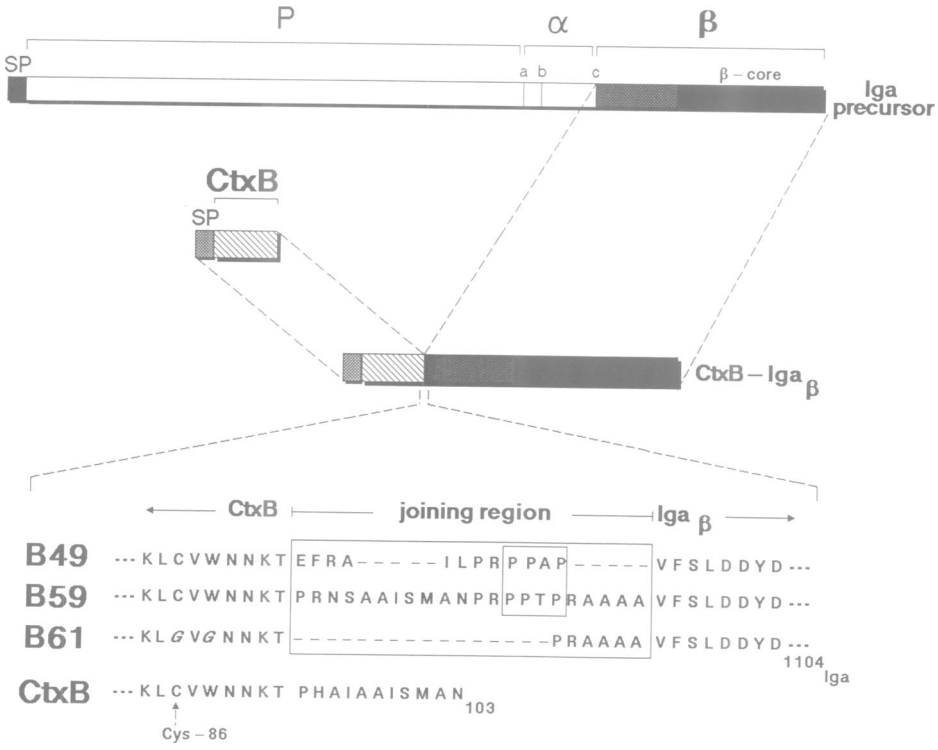

Fig. 2. Linear structure of CtxB−Iga$_\beta$ hybrid proteins. **Top**: 169 kd IgA protease precursor of *N.gonorrhoeae* MS11, including signal peptide (SP), protease domain (P), α-domain (α) and β-domain (β). The β-domain consists of an amino-terminal hydrophilic region (dotted) and the carboxy-terminal β-core region (solid) which harbours the putative transmembrane segments. Sites a, b and c represent autoproteolytic cleavage sites for IgA protease. **Middle**: fusion between CtxB and Iga$_\beta$. **Bottom**: amino acid sequences (one-letter code) encoding the primary structure of the joining regions of CtxB−Iga$_\beta$ hybrid proteins B49, B59 and B61. The point of fusion within the β-region is indicated at amino acid residue D-1104, according to Pohlner *et al.* (1987b). The joining regions of the three hybrids are boxed (large box). The small box specifies cleavage sites for IgA protease contained in proteins B49 and B59. Amino acids substituted for residues Cys86 and Trp88 in protein B61 are printed in italics. The carboxy-terminal portion of native CtxB, comprising 103 amino acids, is shown for comparison.

In this report we show that translational fusion to the β-domain of the IgA protease precursor of *N.gonorrhoeae* MS11 directs export of cholera toxin B subunit, an otherwise periplasmic protein in enterobacteria, to the cell surface of a *gal*E mutant of *S.typhimurium*. We further demonstrate that translocation of the CtxB moiety of the CtxB−Iga$_\beta$ hybrid proteins across the outer membrane requires the passenger protein to be in a 'translocation-competent' conformation.

Results

Construction and expression of ctxB − iga$_\beta$ *gene fusions*

To construct gene fusions of *ctx*B and *iga*$_\beta$ we subcloned the *ctx*B gene from plasmid pCVD002 (Lockman and Kaper, 1983) into the plasmid vector pBA, a derivative of pBR322 (Bolivar *et al.*, 1977). In the resulting plasmid, pTK2 (Figure 1A), *ctx*B is constitutively expressed under the control of an artificial promoter (P_k) which was created by ligation of the insert and vector fragments at the *Cla*I site (Figure 1B). While the 5′ end of the insert DNA contained a −10 promoter region, the 2.4 kb *Cla*I−*Sal*I vector fragment provided a −35 region located between the *Eco*RI and *Cla*I sites of the vector. The position of the artificial expression signal P_k relative to the *ctx*B gene was confirmed by identifying the first nucleotide of the *ctx*B mRNA using primer extension with reverse transcriptase (Klauser, 1987). The promoter structure presented in Figure 1(B) shows 75% homology to the 'consensus promoter' published by Hawley and McClure (1983).

The promoter P_k was also used for the expression of *ctx*B−*iga*$_\beta$ gene fusions. These constructs code for hybrid proteins consisting of CtxB fused at its carboxy terminus to the amino terminus of Iga$_\beta$. The 57 kd CtxB−Iga$_\beta$ hybrid proteins B49, B59 and B61 are encoded by plasmids pTK49, pTK59 and pTK61 respectively (Figures 1A and 2). These proteins appear to be stable and remain cell-associated in *S.typhimurium* G30 recombinants as shown by immunoblot analysis of whole-cell lysates with CtxB-specific antiserum AK55 (Figures 3A, lanes 1, 5 and 9; 5B, arrowhead).

A common feature of the hybrid proteins is the amino-terminal signal peptide of CtxB, which mediates the transport across the inner bacterial membrane. The hybrids are distinct with respect to the joining region between the CtxB and Iga$_\beta$ domains and/or residues 9, 10, 86 and 88 of CtxB (Figure 2): protein B49 lacks 11 carboxy-terminal amino acids of the native CtxB and harbours the original autoproteolytic site 'c' of the Iga precursor of *N.gonorrhoeae* MS11 (Pohlner *et al.*, 1987b). In protein B59, the carboxy terminus of CtxB has been reconstituted, and an artificial cleavage site for IgA protease, Pro-Pro-Thr-Pro (J.Pohlner *et al.*, in preparation), introduced in between the CtxB and Iga$_\beta$ domains. In protein B61, the two Cys residues of the CtxB moiety at positions Cys9 and Cys86 were substituted by Leu and Gly. Furthermore, Trp88, a residue that has been proposed to play a role in the oligomerization process of

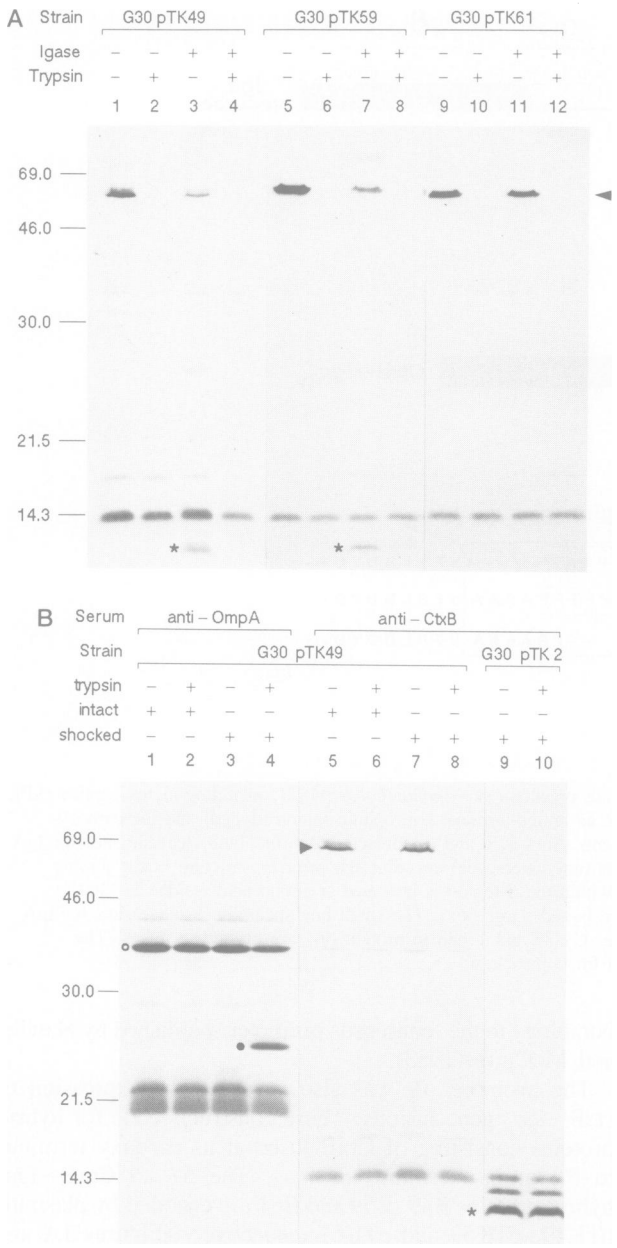

Fig. 3. Protease accessibility analysis of hybrid proteins. (**A**) Immunoblot probed with anti-CtxB serum AK55, showing protease accessibility of intact cells. **Lanes 1–4** show cell lysates of G30(pTK49): (**1**) untreated cells; (**2**) trypsin-treated cells; (**3**) IgA protease-treated cells; (**4**) trypsin treatment of IgA protease-pretreated cells. **Lanes 5–8** show cell lysates of G30(pTK59): (**5**) untreated cells; (**6**) trypsin-treated cells; (**7**) IgA protease-treated cells; (**8**) trypsin treatment of IgA protease-pretreated cells. **Lanes 9–12** show cell lysates of G30(pTK61): (**9**) untreated cells; (**10**) trypsin-treated cells; (**11**) IgA protease-treated cells; (**12**) trypsin treatment of IgA protease-pretreated cells. The arrowhead indicates the 57 kd CtxB–Iga$_\beta$ hybrid proteins. Asterisks specify the 12 kd CtxB proteins generated by IgA protease incubation of G30(pTK49) and G30(pTK59). (**B**) Immunoblots of cell lysates of G30(pTK49) analysed with anti-OmpA serum are shown in **lanes 1–4**: (**1**) intact cells; (**2**) intact cells treated with trypsin; (**3**) shocked cells; (**4**) shocked cells treated with trypsin. Lysates of G30(pTK49) analysed with anti-CtxB serum AK55 are shown in **lanes 5–8**: (**5**) intact cells; (**6**) intact cells treated with trypsin; (**7**) shocked cells; (**8**) shocked cells treated with trypsin. Cell lysates of G30(pTK2) analysed with AK55 are shown in **lanes 9** and **10**: (**9**) shocked cells; (**10**) shocked cells treated with trypsin. Specific protein bands are indicated as follows: CtxB–Iga$_\beta$ hybrid proteins (arrowhead), OmpA protein (open circle), OmpA tryptic fragment (closed circle) and periplasmic CtxB (asterisk).

native CtxB (Ludwig *et al.*, 1985), was replaced by Ala. Glu10 was exchanged for Gly in order to introduce an additional *Xho*I restriction site into the *ctx*B gene, which facilitated screening. The last 10 carboxy-terminal amino acids of CtxB have been deleted in B61, and the protein carries no cleavage site for IgA protease.

Protease accessibility of surface-exposed CtxB

We used various methods to test the targeting behaviour of the CtxB–Iga$_\beta$ hybrids in *S.typhimurium*. A reliable approach that has repeatedly been used to determine the subcellular location of proteins is the accessibility to proteases such as trypsin (Schenkman *et al.*, 1984; Howard and Buckley, 1985). When intact cells are incubated with trypsin, the protease only has access to proteins that reside on the external side of the cell envelope. In osmotically shocked cells, trypsin gains access to the periplasm, and may degrade periplasmic proteins as well as proteins of the cytoplasmic and outer membranes with regions exposed to the periplasm (Freudl *et al.*, 1986; Klose *et al.*, 1988). This approach was used to demonstrate the surface exposure of CtxB by the CtxB–Iga$_\beta$ hybrid proteins B49, B59 and B61 in *S.typhimurium*. Cells containing the appropriate recombinant plasmids, G30(pTK49), G30(pTK59) and G30(pTK61), were incubated with trypsin, as described in Materials and methods. Treated cells were lysed in sample solution, the proteins were separated by SDS–PAGE and then immunoblotted, using the CtxB-specific antiserum AK55. Trypsin treatment of intact cells resulted in the degradation of the immuno-reactive epitopes of proteins B49, B59 and B61 (Figure 3A, lanes 1 and 2, 5 and 6, 9 and 10), indicating that the CtxB moieties of the hybrid proteins were exposed on the bacterial surface.

In a control experiment we tested the integrity of the outer membrane of the recombinant bacterial cells, and the conditions of trypsin treatment. Using the outer membrane protein OmpA as a marker, we showed that the cells expressing and exposing the CtxB–Iga$_\beta$ hybrids were physically intact. In contrast to the CtxB domain, which was removed by trypsin from whole cells (Figure 3B, lanes 5 and 6), the OmpA protein was not accessible (Figure 3B, lanes 1 and 2). However, cleavage by trypsin of both OmpA and of the CtxB hybrids was evident in osmotically shocked cells (Figure 3B, lanes 3 and 4, 7 and 8).

Specific cleavage of surface-exposed hybrid protein by IgA protease

Further surface accessibility studies were performed with purified IgA protease, an enzyme with a narrow cleavage specificity (Pohlner *et al.*, 1987a; J.Pohlner *et al.*, in preparation). Two of the hybrid proteins, B49 and B59, contain cleavage sites for IgA protease, while B61 does not, serving as a control. Proteins B49 and B59 carry the target sequences Pro-Pro-Ala-Pro and Pro-Pro-Thr-Pro respectively (Figure 2). In both hybrid proteins the cleavage site is located in the region linking the CtxB portion with Iga$_\beta$. *S.typhimurium* G30 cells producing the proteins B49, B59 and B61 were incubated with purified IgA protease. Cell lysates were separated by SDS–PAGE and analysed by immunoblotting with the CtxB-specific antiserum AK55. The hybrid proteins B49 and B59 were cleaved by IgA protease (Figure 3A, lanes 3 and 7), while B61 was not (Figure 3A, lane 11).

Unexpectedly, the 12 kd CtxB cleavage products of B49

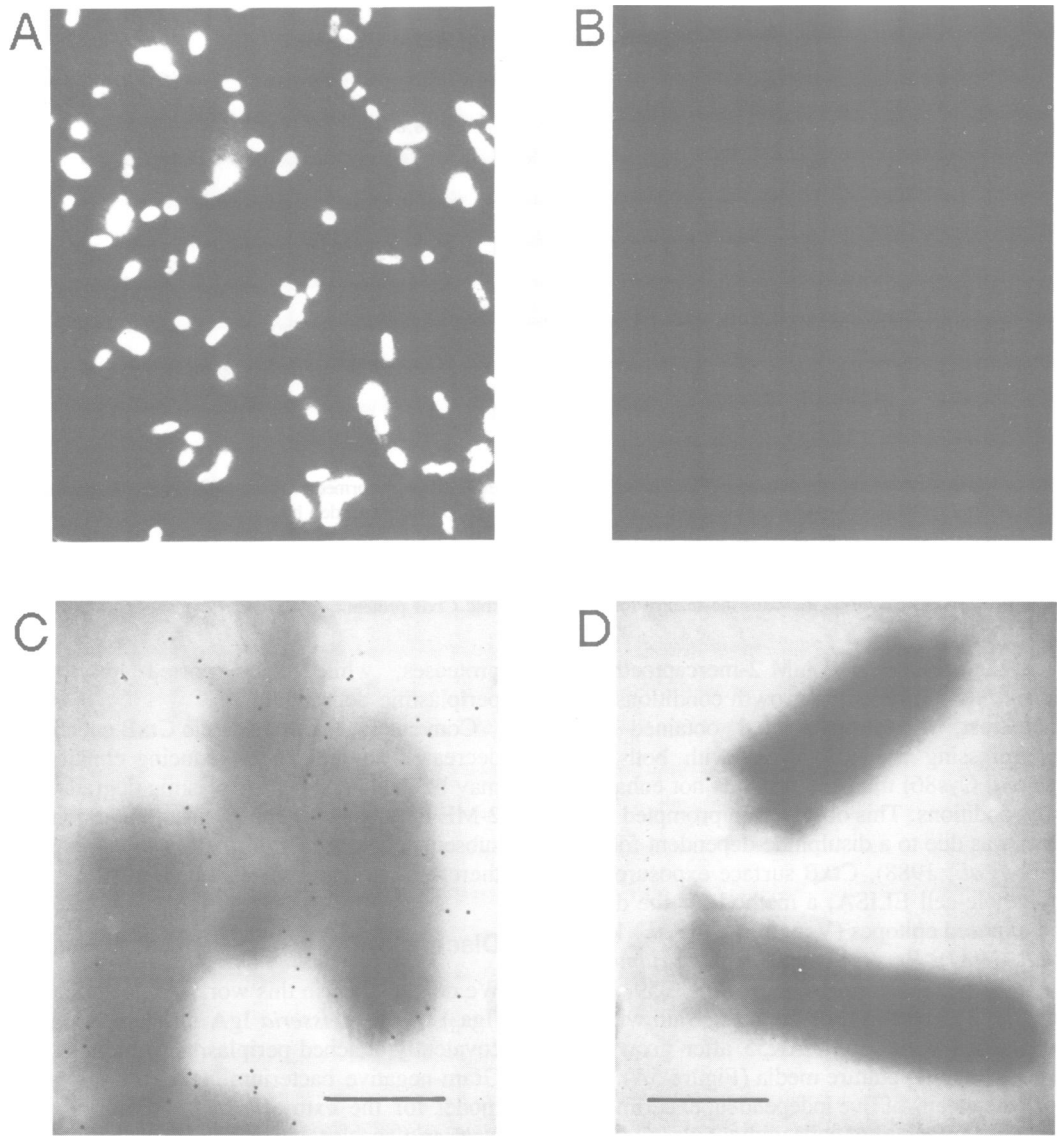

Fig. 4. Immunofluorescence and immunogold labelling of CtxB in *Salmonella*. (**A** and **B**) Indirect immunofluorescence analysis using anti-CtxB serum AK55 of *S.typhimurium* harbouring plasmid pTK49 and the control plasmid pTK2 respectively. 2000×. (**C** and **D**) Electron micrographs (32 000×; scale bars = 0.5 μm) of AK55 and immunogold-labelled intact *S.typhimurium* harbouring plasmid pTK61 and control plasmid pTK2 respectively.

and B59 were not released into the supernatant but remained cell-associated (see asterisks in Figure 3A). However, subsequent degradation of these cleavage products by externally added trypsin clearly demonstrates their surface localization (Figure 3A, lanes 3 and 4, 7 and 8). We assume that after outer membrane translocation, the propagated CtxB proteins interact with a surfaced structure of *S.typhimurium* G30.

In addition to verifying the surface localization, trypsin sensitivity suggests a different conformation of surface-exposed CtxB versus the periplasmic CtxB. Periplasmic CtxB produced by the control clone G30(pTK2) is resistant to trypsin digestion (Figure 3B, lanes 9 and 10). This can be attributed to pentamer formation (Hirst and Holmgren, 1987b).

Immunolabelling of cell surface exposed CtxB

Indirect immunofluorescence microscopy and immunogold electron microscopy of intact cells was used to test whether the CtxB−Iga$_\beta$ hybrids on the surface of *Salmonella* react with CtxB-specific antibodies. Cells of *S.typhimurium* G30 containing plasmids pTK49, pTK59, pTK61, or the control plasmid pTK2, were exposed to antiserum AK55. Cell-bound antibody was detected either by FITC-labelled goat anti-rabbit IgG or protein A−gold, as appropriate. Intact cells synthesizing proteins B49, B59 and B61 were positive by both methods (Figure 4A and C), demonstrating that the CtxB parts of the fusion proteins were accessible to antibodies. An average of 10−15 gold particles were counted on G30(pTK49), 10−15 particles on G30(pTK59) and 20−25 particles on G30(pTK61). The control clone, G30(pTK2) harbouring periplasmic CtxB, was negative in both the immunofluorescence and immunogold labelling experiments (Figure 4B and D).

Influence of protein structure on the translocation of CtxB across the outer membrane

Interestingly, immunofluorescence signals obtained from G30(pTK49 and pTK59) were intensified when the cells

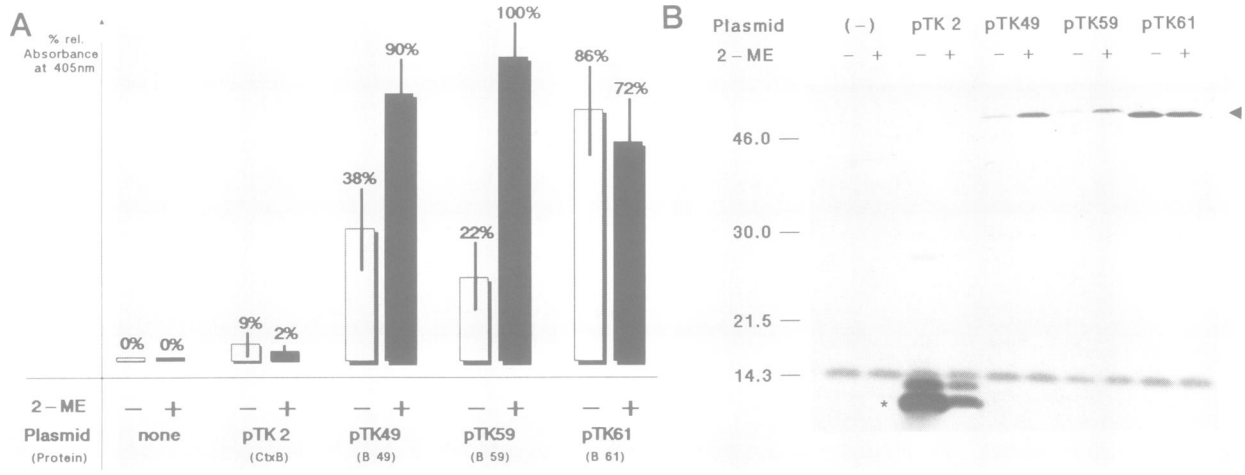

Fig. 5. Influence of CtxB structure on translocation competence. (**A**) Whole-cell ELISA performed with *S.typhimurium* either without plasmid or containing recombinant plasmids pTK2, pTK49, pTK59 or pTK61. Corresponding proteins encoded by these plasmids are designated CtxB, B49, B59 or B61. The cells were grown in the presence (+) or absence (−) of 5 mM 2-mercaptoethanol (2-ME). Results are presented as % relative absorbance at 405 nm with the highest value as 100%. (**B**) Immunoblot of *S.typhimurium* whole-cell lysates probed with CtxB-specific antiserum AK55. Cells, culture conditions, designations and the order of the samples are as described for the whole-cell ELISA. The arrowhead specifies CtxB−Iga$_\beta$ hybrid proteins. The asterisk indicates the mature form of periplasmic CtxB protein.

were cultured in the presence of 5 mM 2-mercaptoethanol (2-ME), compared to non-reducing growth conditions (not shown). In contrast, the strong signal obtained with G30(pTK61) expressing a CtxB fusion with both Cys residues (Cys9 and Cys86) mutagenized was not enhanced under reducing conditions. This observation prompted us to test if this effect was due to a disulphide-dependent folding of CtxB (Hardy *et al.*, 1988). CtxB surface exposure was quantitated by whole-cell ELISA, a method for the detection of surface-exposed epitopes (Van der Ley *et al.*, 1985; Agterberg *et al.*, 1987). Recombinant *S.typhimurium* G30 producing the CtxB−Iga$_\beta$ hybrid proteins B49, B59, B61, the periplasmic CtxB (Figure 2), or the host strain without plasmid were assayed with serum AK55 after growth in reducing and non-reducing culture media (Figure 5A). The values given are the means of five independent experiments; the background level (reaction of cells without plasmid) was subtracted and the highest absorbance was set as 100%. The hybrid proteins B49 and B59 each contain two Cys residues in the CtxB moiety. G30 cells producing these hybrids showed an increase in the ELISA signal when grown in medium supplemented with 2-ME up to 5-fold. Cells producing mutant protein B61, which does not contain cysteines in CtxB, reacted equally strongly in the presence or absence of 2-ME. Control cells, containing plasmid pTK2, consistently gave background values under both culture conditions (Figure 5A).

In addition to whole-cell ELISA, bacterial cell lysates were analysed by immunoblotting, using antiserum Ak55 (Figure 5B). This was to demonstrate that the addition of 2-ME does not affect the expression of CtxB, but instead increases the stability of CtxB as a result of its translocation across the outer membrane. Consistently the amount of control protein B61 (containing a Leu9 and a Gly86) was unchanged in the presence of 2-ME. The amounts of the hybrids B49 and B59 (both containing a Cys9 and a Cys86), however, were increased in the presence of 2-ME (Figure 5B). Hence the lower levels of the latter hybrid proteins detected under non-reducing conditions probably result from the degradation of the non-translocated proteins by periplasmic proteases, while the exported hybrid CtxB escapes periplasmic degradation.

Conversely, for periplasmic CtxB encoded on pTK2, the decreased amount under reducing conditions (Figure 5B) may be explained by periplasmic degradation. In this case 2-ME may partially inhibit disulphide bond formation and subsequent pentamer formation of periplasmic CtxB, and therefore decrease its resistance to proteases.

Discussion

We demonstrate in this work the potential of the β-domain (Iga$_\beta$) of the *Neisseria* IgA protease precursor to direct a covalently attached periplasmic protein to the surface of a Gram-negative bacterium. Our data support the previous model for the extracellular secretion of IgA protease in *Neisseria* species and *E.coli*, and confirm the central role of Iga$_\beta$, subregion of the previously described 'helper' domain (Pohlner *et al.*, 1987b), in outer membrane transport. The use of Iga$_\beta$ fusions for export of proteins from Gram-negative bacteria has a variety of future applications: (i) the extracellular production of medically and biologically relevant proteins from *E.coli*, (ii) the exposition of single or multiple epitopes, as well as complete proteins on the cell surface of enterobacteria for medical diagnostics, (iii) the construction of recombinant live oral vaccines based on attenuated *Salmonella* strains and (iv) the availability of a new system to study specific topics of membrane translocation of proteins *in vivo* and *in vitro*.

Here we constructed translational *iga* gene fusions in which the regions coding for the mature IgA protease and the α-protein were replaced by the *ctx*B gene from *V.cholerae* (Lockman and Kaper, 1983). We analysed the transport properties of the hybrid gene products using *S.typhimurium* G30 as a host (Osborn *et al.*, 1964). The CtxB−Iga$_\beta$ fusion proteins B49, B59 and B61 were found to be integrated in the outer membrane of *Salmonella*. In addition, the CtxB moieties of the hybrids were exposed on the bacterial surface as shown by indirect immunofluorescence (Figure 4A), immunogold labelling (Figure 4C)

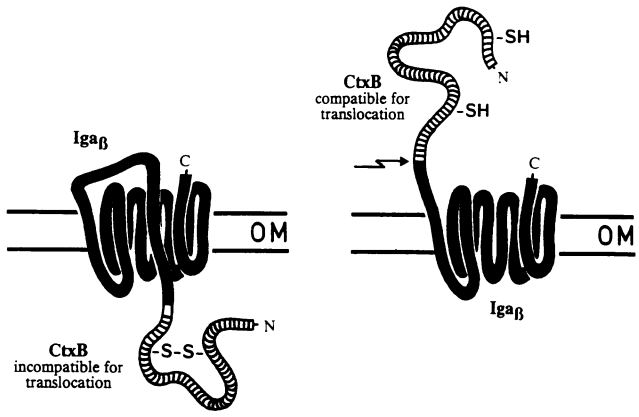

Fig. 6. Model of β-domain mediated exposition of CtxB on the surface of *Salmonella*. CtxB−Iga$_\beta$ hybrid proteins assemble in the outer membrane (OM) using the Iga$_\beta$ function. **Left scheme**: the conjugated CtxB portion undergoes a conformational change by the formation of a disulphide bond between residues Cys9 and Cys86 at the inner side of the outer membrane. This bond induces a globular structure that cannot be translocated through the putative membrane pore formed by Iga$_\beta$. The CtxB moiety residing at the periplasmic side of the membrane is susceptible to degradation by host proteases. **Right scheme**: Addition of sulphydryl reagents, such as 2-ME, prevents disulphide bond formation, allowing the CtxB to be translocated to the external surface of the membrane. The arrow indicates the position of the IgA protease cleavage site.

and accessibility to the enzymes trypsin and IgA protease (Figure 3). We demonstrated that the efficiency of outer membrane translocation was increased under reducing culture conditions.

This latter finding suggests that the translocation step of covalently attached CtxB across the outer membrane is dependent on its conformation. Native CtxB and the homologue EtxB from enterotoxigenic *E.coli* strains form intramolecular disulphide bonds prior to subunit assembly in the periplasm (Hardy *et al.*, 1988). We believe that disulphide bond formation also occurs in CtxB−Iga$_\beta$ fusions, thereby blocking the translocation event. Growth of the cells in medium containing the sulphydryl reagent 2-mercaptoethanol (2-ME), enhanced translocation of the CtxB portion several-fold (Figure 5A). We propose that 2-ME penetrates the cell envelope and prevents the CtxB moiety from forming intramolecular disulphide bonds. Since the two Cys residues of CtxB lie far apart (Cys9 and Cys86) we assume that the formation of an intramolecular disulphide bond creates a globular structure unable to cross the membrane. Instead this structure becomes trapped at the periplasmic face of the outer membrane. In the presence of 2-ME, disulphide bond formation is prevented, allowing the CtxB moiety to be translocated across the membrane (see Figure 6).

Our interpretation of the effect of 2-ME on the translocation of CtxB is confirmed by the substitution of both Cys residues Cys9 and Cys86 with leucine and glycine residues. The CtxB portion of the mutant fusion protein B61 was translocated through the outer membrane independent of 2-ME. Two other mutant proteins, B62 and B63, with only one Cys residue each (Cys86 or Cys9), are similarly competent for membrane translocation, regardless of the presence or absence of 2-ME (data not shown). These data indicate that intramolecular disulphide bond formation renders the wild-type CtxB portion translocation-incompetent.

The observation that the fusion proteins B49 and B59 appear as weaker bands in the immunoblot when the strains were cultured without 2-ME (Figure 5B) suggests that the fusions are prone to degradation by host proteases, if they are retained in the periplasm. Translocation to the cell surface thus seems to prevent the proteins from being degraded. The Iga$_\beta$-mediated translocation of CtxB through the outer membrane therefore competes with periplasmic protein folding and degradation. A similar competition probably exists in the natural situation for the translocation of the IgA protease domain, since there is no accumulation of intracellular forms when outer membrane transport is impaired (Pohlner *et al.*, 1987b). In this regard it is interesting that the whole IgA protease precursor (Iga), which consists of ~ 1500 amino acids, contains only two Cys residues, 11 amino acids apart from each other. The Iga precursor is therefore unlikely to form stable disulphide bond-induced tertiary structures that could compete with the translocation process.

The transport of CtxB−Iga$_\beta$ hybrid proteins to the outer membrane is clearly a function of the outer membrane targeting and membrane assembly functions of Iga$_\beta$. As will be shown elsewhere, Iga$_\beta$ integrates into the membrane, forming a characteristic structure irrespective of the fusion partner or the host cell strain. The integral portion of Iga$_\beta$, the β-core, exhibits an amphipathic β-sheet conformation (Pohlner *et al.*, 1987b; F.Jähnig, unpublished). As a common feature, amphipathic β-sheet structures are characteristic of many outer membrane proteins (Vogel and Jähnig, 1986; Klose *et al.*, 1988; Misra and Benson, 1988; Tommassen, 1988). Such proteins have successfully been used as carriers for antigenic determinants or peptides up to 21 amino acids in size without disturbing correct membrane assembly (Charbit *et al.*, 1986; Agterberg *et al.*, 1987; Freudl, 1989). In this regard, Iga$_\beta$ can be considered as an outer membrane protein with potentially similar characteristics and applications. In addition, however, with Iga$_\beta$ it is possible to target even large amino-terminally attached polypeptides onto the cell surface of Gram-negative bacteria, as evidenced by the export of CtxB (as well as IgA protease in the natural situation).

None the less, transport of foreign polypeptides across the outer membrane utilizing the Iga$_\beta$ shuttle may be limited to a distinct class of proteins. These proteins have to satisfy the functional and structural requirements for leader-peptide-dependent transport across the inner membrane. A general requirement is the maintenance of a translocation-competent conformation prior to transport that may be distinct from the final protein structure. This prerequisite applies to proteins transported across eukaryotic membrane systems (Eilers and Schatz, 1986; Chirico *et al.*, 1988; Eilers *et al.*, 1988) and to bacterial inner membranes (Randall and Hardy, 1986; Bochkareva *et al.*, 1988; Collier *et al.*, 1988; Crooke *et al.*, 1988). The same constraints probably apply for the Iga$_\beta$-mediated transport through the outer membrane of Gram-negative bacteria since folding of the cholera toxin B subunit apparently interferes with its outer membrane translocation.

Materials and methods

Bacterial strains and growth conditions

E.coli strain GC1 (K12$_{r-m+}$ MDU; Meyer *et al.*, 1982) was used for propagation of plasmid DNA. *S.typhimurium* G30A (Osborn *et al.*, 1964) was

Table I. Synthetic oligonucleotides used in this study

Name	Usage[a]	Length (bp)	Sequence (5′ to 3′)
TK002	DNA sequencing (+)	17	GCGATTGAAAGGATGAA
TK003	RNA sequencing (−)	19	CATAATTCATCCTTAATTC
TK006	Cloning (+)	46	AATTCAGCCGCAATTAGTATGGCAAATCCACGTCCACCAACACCGC
TK007	Cloning (−)	40	GGTGTTGGTGGACGTGGATTTGCCATACTAATTGCGGCTG
TK015	PCR (+)	56	CAGCATATGCACATGGAACACCTCAAAATATTACTGATTTGCTCGAGGAATCACAC
TK016	PCR (−)	51	TTAAGAATTCCGCGGCGTTTTATTATTCCCTACACCTAACTTTTCGACTTT

[a](+) and (−) refer to coding and complementary strand sequences in the *ctx*B gene respectively.

from the strain collection of P.A. Manning. Transformation of *S.typhimurium* with plasmid DNA was achieved by the $MgCl_2/CaCl_2$ method (Lederberg and Cohen, 1974). *S.typhimurium* was cultured with aeration in Luria−Bertani broth (LB broth) at 37°C. The culture medium was supplemented with 100 μg/ml ampicillin for plasmid containing strains. 2-ME (5 mM) was added as indicated.

Plasmid construction

The *ctx*B gene was subcloned from plasmid pCVD002 (Lockman and Kaper, 1983) by inserting the 1.6 kb *ClaI*−*SalI* fragment into the corresponding restriction sites of cloning vector pBA. The single *Eco*RI site of this plasmid pTK1 was filled in with Klenow fragment of DNA polymerase and ligated to 10mer *KpnI* linkers to obtain plasmid pTK2. In order to facilitate the construction of hybrid *ctx*B−*iga*$_\beta$ genes, *Eco*RI linkers were introduced into the 3′ region of the *ctx*B open reading frame. For this purpose plasmid pTK2 was linearized with *Bst*XI (a restriction site in the terminator region of the *ctx*B gene) and subjected to *Bal*31 exonuclease digestion followed by Klenow fill-in reaction, and then ligation to 10mer *Eco*RI linkers. The position of *Eco*RI linkers in plasmids pTK18 and pTK23 was confirmed by double-stranded DNA sequencing (Chen and Seeburg, 1985) using oligonucleotide TK002 (Table I) as primer. Plasmid pTK49 was obtained by ligating the *XhoI*−*SalI* fragment from plasmid pIP503, encoding the *iga*$_\beta$ gene, to the 2.9 kb *Eco*RI−*SalI* vector fragment of plasmid pTK18. The cohesive ends of the *XhoI* and *Eco*RI sites of pIP503 and pTK18 were filled in using Klenow prior to ligation. Plasmid pIP503, a derivative of pIP100 (Halter *et al.*, 1984), contains a *XhoI* site at position 3441 of the *iga* gene (Pohlner *et al.*, 1987b), which was originally introduced by site-directed mutagenesis (J.Pohlner, unpublished). Plasmid pTK59 resulted from the ligation of the *PvuI*−*Eco*RI fragment of plasmid pTK23 with the *PvuI*−*Sac*II fragment of pEx38.2 (J.Scheurlen, unpublished), in the presence of annealed, complementary oligonucleotides TK006 and TK007. Plasmid pEx.38.2 contains a *Sac*II site at position 3455 of the *iga* gene (Pohlner *et al.*, 1987b). Plasmid pTK61 was obtained by ligating the *NdeI*−*Sac*II fragment of pTK59 to a mutated *ctx*B gene contained on a *NdeI*−*Sac*II fragment, which was synthesized by polymerase chain reaction (PCR) (Saiki *et al.*, 1988). For PCR, 5 ng of linearized plasmid pTK2 was used as template and 100 pmol of oligonucleotides TK015 and TK016 served as primers. The reaction was performed in a thermal cycler using *Taq* polymerase (Perkin-Elmer). DNA cloning was performed essentially as described by Maniatis *et al.* (1982). Oligonucleotides were synthesized on a MilliGen 7500 DNA synthesizer.

Localization of transcription initiation signals

For constitutive expression of the *ctx*B gene and *ctx*B−*iga*$_\beta$ hybrid genes, the chimeric promoter P_k was used. The position of the promoter signal relative to the genes was determined by RNA primer extension using MLV reverse transcriptase. Primer extension was performed according to the published RNA sequencing protocol (Stern *et al.*, 1986) except that 200 μM of all four dNTPs were added to the reaction mixture and dideoxynucleotides were excluded. 5′-End-labelled oligonucleotide TK003 served as primer for the extension reaction. RNA isolation from *E.coli* was performed as described (Chirgwin *et al.*, 1979).

Immunofluorescence

S.typhimurium was cultured in 2-ME-supplemented medium. Cells were harvested and fixed with 1% paraformaldehyde/0.05% glutaraldehyde in PBS for 15 min at 21°C followed by 15 min on ice. They were then twice washed with PBS and incubated with CtxB-specific rabbit antiserum AK55 (1:4 dilution) for 1 h at 4°C. Cells were washed three times with PBS and incubated with FITC-labelled goat anti-rabbit IgG (1:100 dilution) for 30 min at 4°C. After further washing, cells were resuspended in PBS/Citifluor 1:1 (Citifluor, London) and coated onto glass slides by centrifugation. Slides were examined under a Nikon Microphot-Fx microscope, and photographs taken with Kodak colour slide film.

Immunogold electron microscopy

The procedure was performed as described previously (Tommassen *et al.*, 1985) with some modifications. Cells were cultured in 2-ME-supplemented medium and fixed as above. The cells were washed three times with PBS, floated on formvar-coated grids and incubated successively with rabbit anti-CtxB serum (1:4 dilution) and protein A−gold complexes. In order to visualize gold particles (10 nm), cells were not counter-stained.

Protease treatment of whole cells

One millilitre of a late logarithmic phase culture of *S.typhimurium* was washed twice with TBS (20 mM Tris, pH 7.5, 140 mM NaCl), then resuspended in 1 ml of TBS. One hundred microlitres of the cell suspension was incubated either with trypsin (500 μg/ml final concentration) for 2 h on ice, or with 5 μl of purified IgA protease (0.5 μg) for 2 h at 37 °C. Cells treated with both proteases were first incubated with IgA protease for 2 h at 37 °C, then trypsin was added and the mixture was kept on ice for 30 min. Cells were osmotically shocked by mixing 100 μl of the cell suspension with 100 μl of 40% sucrose in TBS−EDTA solution (20 mM Tris, pH 7.5, 140 mM NaCl, 20 mM EDTA) prior to incubation with trypsin (500 μg/ml) on ice for 2 h. Trypsin digestion was stopped by adding an equimolar amount of trypsin inhibitor. Cells or spheroplasts were sedimented by centrifugation, the supernatant was discarded and 100 μl of sample solution was added. Samples were then boiled for 5 min and sonicated briefly to disrupt DNA. Aliquots (5−10 μl) were loaded on 12.5 or 17.5% SDS−polyacrylamide gels (Laemmli, 1970) and then analysed by immunoblot as described below.

Preparation of whole-cell lysates from Salmonella

Cells were washed once with PBS and resuspended in reducing sample solution. The suspension was boiled for 5 min and sonicated briefly to disrupt DNA. Five microlitres of each sample corresponding to 50 μl of liquid culture, was analysed by SDS−PAGE and immunoblot.

Immunoblot

Proteins separated on SDS−PAGE were transferred onto nitrocellulose at 1 mA/cm^2 for 1.5 h using a semi-dry blot system (Biotec Fischer). The filters were saturated with 3% BSA in TBS (20 mM Tris−HCl, pH 7.5, 140 mM NaCl) for 1 h followed by overnight incubation with CtxB-specific rabbit antiserum AK55 (1:100 dilution). The nitrocellulose was washed three times in TTBS (TBS containing 0.5% Tween-20) and incubated with alkaline phosphatase-conjugated protein A (1:1000 dilution). The nitrocellulose was washed three times with TTBS, once with 100 mM Tris−HCl (pH 9.6) and incubated with 5-bromo-4-chloro-3-indoxyl-phosphate (0.1 μg/ml final concentration) and nitroblue tetrazolium (0.5 μg/ml final concentration) in 100 mM Tris−HCl (pH 9.6).

Whole-cell ELISA

The procedure was performed as published (Van der Ley *et al.*, 1985) with minor modifications. *Salmonella* were cultured in the presence or absence of 5 mM 2-ME. After washing twice, the cells were resuspended in PBS to the original optical density of the culture and serial dilutions transferred to microtitre plates (Nunc Immuno Plate F; 100 μl/well). Cells were immobilized by drying at 37°C overnight. Additional binding sites on the plastic matrix were blocked with 2% BSA (150 μl/well) for 2 h at 37°C. Incubation with CtxB-specific antiserum (1:100 dilution; 100 μl/well) was then performed for 1 h at 37°C. Cells were washed eight times with PBS (200 μl/well) and incubated with alkaline phosphatase-conjugated protein (1:1000 dilution 100 μl/well) for 30 min at 37°C. After further washing with PBS, the substrate *p*-nitro-phenyl-phosphate (p-NPP) in 0.1%

ethanolamine (pH 10.3) was added. Absorbance was measured at 405 nm using an ELISA reader EAR 400 (SLT-Lab instruments). All antibody dilutions were made in PBS without detergent.

Materials

Enzymes were purchased from Boehringer, trypsin inhibitor was from Serva. Protein A–gold was from Plano and DNA linkers from Pharmacia. Chemicals were from Sigma, Serva and Merck.

Acknowledgements

We would like to thank Dr U.Henning for the generous gift of antiserum to OmpA protein, and W.Eisenbeiß for valuable advice in electron microscopy. We thank Dr B.D.Robertson for critical suggestions on the manuscript, and C.Müller for photography.

References

Agterberg,M., Adriaanse,H. and Tommassen,J. (1987) *Gene*, **59**, 145–150.
Bochkareva,E.S., Lissin,N.M. and Girshovich,A.S. (1988) *Nature*, **336**, 254–257.
Bolivar,F., Rodriguez,R.L., Greene,P.J., Betlach,M.C., Heyneker,H.L. and Boyer,H.W. (1977) *Gene*, **2**, 95–113.
Charbit,A., Boulain,J.C., Ryter,A. and Hofnung,M. (1986) *EMBO J.*, **5**, 3029–3037.
Chen,E.Y. and Seeburg,P.H. (1985) *DNA*, **4**, 165–170.
Chirgwin,J.M., Przbyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) *Biochemistry*, **18**, 5294–5299.
Chirico,W.J., Waters,M.G. and Blobel,G. (1988) *Nature*, **332**, 805–810.
Collier,D.N., Bankaitis,V.A., Weiss,J.B. and Bassford,P.J. (1988) *Cell*, **53**, 273–283.
Crooke,E., Guthrie,B., Lecker,S., Lill,R. and Wickner,W. (1988) *Cell*, **54**, 1003–1011.
Eilers,M. and Schatz,G. (1986) *Nature*, **322**, 228–232.
Eilers,M., Hwang,S. and Schatz,G. (1988) *EMBO J.*, **7**, 1139–1145.
Freudl,R. (1989) *Gene*, **82**, 229–236.
Freudl,R., Schwarz,H., Stierhof,Y.-D., Gamon,K., Hindennach,I. and Henning,U. (1986) *J. Biol. Chem.*, **261**, 11355–11361.
Halter,R., Pohlner,J. and Meyer,T.F. (1984) *EMBO J.*, **3**, 1595–1601.
Halter,R., Pohlner,J. and Meyer,T.F. (1989) *EMBO J.*, **8**, 2737–2744.
Hardy,S.J.S., Holmgren,J., Johansson,S., Sanchez,J. and Hirst,T.R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7109–7113.
Hawley,D.K. and McClure,W.R. (1983) *Nucleic Acids Res.*, **11**, 2237–2255.
Hirst,T.R. and Holmgren,J. (1987a) *J. Bacteriol.*, **169**, 1037–1045.
Hirst,T.R. and Holmgren,J. (1987b) *Proc. Natl. Acad. Sci. USA*, **84**, 7418–7422.
Holmgren,J. (1981) *Nature*, **292**, 413–417.
Howard,S.P. and Buckley,J.T. (1985) *J. Bacteriol.*, **161**, 1118–1124.
Klauser,T. (1987) Diploma thesis, Technische Universität, München.
Klose,M., MacIntyre,S., Schwarz,H. and Henning,U. (1988) *J. Biol. Chem.*, **263**, 13297–13302.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lederberg,E.M. and Cohen,S.N. (1974) *J. Bacteriol.*, **119**, 1072–1074.
Lockman,H. and Kaper,J.B. (1983) *J. Biol. Chem.*, **258**, 13722–13726.
Ludwig,D.S., Holmes,R.K. and Schoolnik,G.K. (1985) *J. Biol. Chem.*, **260**, 12528–12534.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mekalanos,J.J., Swartz,D.J. and Pearson,G.D.N. (1983) *Nature*, **306**, 551–557.
Meyer,T.F., Mlawer,N. and So,M. (1982) *Cell*, **30**, 45–52.
Meyer,T.F., Halter,R. and Pohlner,J. (1987) In McGhee,J.R., Mestecky,J., Ogra,P.L. and Bienenstock,J. (eds), *Recent Advances in Mucosal Immunology*. Plenum Press, New York, Vol. 216B, pp. 1271–1281.
Misra,R. and Benson,S.A. (1988) *J. Bacteriol.*, **170**, 3611–3617.
Osborn,M.J., Rosen,S.M., Rothfield,L., Felemick,L.D. and Horecker,B.L. (1964) *Science*, **145**, 783.
Pearson,G.D.N. and Mekalanos,J.J. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2976–2980.
Pohlner,J., Halter,R. and Meyer,T.F. (1987a) *Antonie van Leeuwenhoek*, **53**, 479–484.
Pohlner,J., Halter,R., Beyreuther,K. and Meyer,T.F. (1987b) *Nature*, **325**, 458–462.
Pohlner,J., Langenberg,U. and Meyer,T.F. (1990) *Bacterial Protein Toxins*. Gustav Fischer Verlag, Stuttgart, in press.
Randall,L.L. and Hardy,S.J.S. (1986) *Cell*, **46**, 921–928.
Ribi,H.O., Ludwig,D.S., Mercer,K.L., Schoolnik,G.K. and Kornberg,R.D. (1988) *Science*, **239**, 1272–1276.
Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B. and Erlich,H.A. (1988) *Science*, **239**, 487–494.
Schenkman,S., Tsugita,A., Schwartz,M. and Rosenbusch,J.P. (1984) *J. Biol. Chem.*, **259**, 7570–7576.
Stern,A., Brown,M., Nickel,P. and Meyer,T.F. (1986) *Cell*, **47**, 61–71.
Tommassen,J., Leunissen,J., Damme-Jongsten,M. and Overduin,P. (1985) *EMBO J.*, **4**, 1041–1047.
Tommassen,J. (1988) In Op den Kamp,J.A.F. (ed.), *Membrane Biogenesis*, NATO ASI series, Vol. 16. Springer-Verlag, Berlin, pp. 351–373.
Van der Ley,P., Amesz,H., Tommassen,J. and Lugtenberg,B. (1985) *Eur. J. Biochem.*, **147**, 401–407.
Vogel,H. and Jähnig,F. (1986) *J. Mol. Biol.*, **190**, 191–199.

Received on March 12, 1990