Expression of the B subunit of *Escherichia coli* heat-labile enterotoxin in a marine *Vibrio* and in a mutant that is pleiotropically defective in the secretion of extracellular proteins

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A marine *Vibrio* (designated *Vibrio* sp. 60) that is related to *Vibrio anguillarum* was used as a host for a plasmid that encodes the non-toxic B subunit (EtxB) of *Escherichia coli* heat-labile enterotoxin. Expression of EtxB in *Vibrio* sp. 60 resulted in the efficient and selective secretion of the B subunit into the extracellular growth medium. This indicated that *Vibrio* sp. 60, which does not normally produce cholera-like enterotoxins, nonetheless possesses a secretory machinery that permits these toxins to be translocated across its cytoplasmic and outer membranes. Expression of EtxB in a *sec* mutant of *Vibrio* sp. 60 (MVT1192), which had previously been shown to be defective in the secretion of several extracellular proteins, resulted in approximately 95% of the B subunit remaining entrapped within the periplasm of the bacterial cell envelope. This implies that the mutation in MVT1192 defines a locus that determines a common step in the secretion of extracellular proteins, including oligomeric toxins.

Introduction

Bacterial species belonging to the genus *Vibrio* exhibit a remarkable capacity to secrete proteins through their double-membraned envelopes into the surrounding medium (Finkelstein & LoSpalluto, 1970; Oishi *et al.*, 1979; Young & Broadbent, 1982; Neill *et al.*, 1983a; Hirst *et al.*, 1984a; Nishibuchi & Kaper, 1985; Mercurio & Manning, 1985; Focareta & Manning, 1987; Ichige *et al.*, 1988; Hirst & Welch, 1988). Many of the secreted proteins produced by pathogenic vibrios are important virulence factors, including cholera enterotoxin, haemolysins and neuraminidases, etc., which are secreted, for example by *Vibrio cholerae*. The molecular mechanism of protein secretion by vibrios is as yet only poorly understood; nonetheless, studies on secretion of enterotoxins by *V. cholerae* have revealed that two independent translocation events are involved (Hirst *et al.*, 1984a; Hirst & Holmgren, 1987a,b; Hirst, 1991). The first of these encompasses the export of the toxin subunits across the bacterial cytoplasmic membrane. This involves the production of the toxin subunits from a polycistronic

mRNA that encodes separate precursor A and B subunits that carry typical amino-terminal signal sequences, which are cleaved off during the export process. This is followed by the liberation of the mature subunits into the periplasm, where they assemble into a holotoxin complex comprised of one A subunit and five B subunits. The second translocation event involves the transfer of the toxin across the bacterial outer membrane by an apparently selective mechanism that permits the secretion of the toxin into the medium but prevents the release of resident periplasmic proteins.

A similar two-step mechanism may operate for the secretion of other extracellular proteins from vibrios. The proteins that have so far been characterized are synthesized with an amino-terminal signal sequence which would facilitate export across the cytoplasmic membrane into the periplasm, prior to their secretion across the outer membrane (Mekalanos *et al.*, 1983; Mercurio & Manning, 1985; Focareta & Manning, 1987; Vimr *et al.*, 1988). Several attempts have been made to analyse the mechanism of protein secretion in *Vibrio* spp., chiefly through the isolation of mutants which show a phenotypic defect in secretion that results in the accumulation of secreted proteins in the periplasm. A *V. cholerae* mutant that exhibited this phenotype, M14, was isolated by Holmes and coworkers (1975) and had high

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levels of cell-associated cholera toxin. Fractionation experiments showed that the cell-associated toxin indeed accumulates within the periplasm (Hirst & Holmgren, 1987a). More recently, Ichige *et al.*, (1988), described the isolation of several mutants of a non-pathogenic marine *Vibrio* (designated *Vibrio* sp. 60) which exhibits a pleiotropic *sec* defect in the secretion of extracellular proteins. Studies by Buckley and coworkers on the expression of aerolysin from *Aeromonas hydrophila* in both *Vibrio* sp. 60 and its *sec* mutants revealed that the protein was secreted from the wild-type but not from the *sec* strains (Wong *et al.*, 1990). As yet, the nature of the genetic defects in either *V. cholerae* M14 or *Vibrio* sp. 60 mutants remains unknown.

This paper describes the heterologous expression of the B subunit of heat-labile enterotoxin (EtxB) from *Escherichia coli* (which is structurally and functionally related to the B subunit of cholera toxin) in the wild-type *Vibrio* sp. 60 strain and in a mutant that is pleiotropically defective in the secretion of extracellular proteins.

Methods

Bacterial strains and plasmids. *Vibrio* sp. 60 (strain MVT606) and a secretion-defective mutant of *Vibrio* sp. 60 (strain MVT1192) were obtained from Dr A. Ichige, University of Tokyo, Japan (Ichige *et al.*, 1988). Both strains were plated onto Luria agar (Miller, 1974) supplemented with an additional 1% (w/v) NaCl and 50 µg rifampicin ml⁻¹. A spontaneous rifampicin-resistant mutant of each strain was isolated and used for all subsequent experiments. *E. coli* S17-1 (RP4-2-Tc::Mu-Km::Tn7, Tp Sm), *pro r⁻m⁺* which contains RP4 integrated into the chromosome (Simon *et al.*, 1983) was used for mobilizing plasmids into *Vibrio* sp. 60.

Plasmid pMMB68 (Sandkvist *et al.*, 1987) is a derivative of the broad-host range plasmid RSF1010 (Furste *et al.*, 1986) and contains the gene for the B subunit of heat-labile enterotoxin from *E. coli* H74-114 of human origin (Dallas, 1983; Leong *et al.*, 1985). The plasmid encodes resistance to β-lactams. Plasmid pMMB25, also derived from RSF1010 carries the gene encoding catechol 2,3-dioxygenase (*xylE*) from *Pseudomonas putida* (Bagdasarian *et al.*, 1983; Hirst & Holmgren, 1987a). Plasmids pMMB68 or pMMB25, which had previously been transformed into *E. coli* S17-1, were mobilized into the rifampicin-resistant derivatives of MVT606 and MVT1192. This was carried out by mixing exponential-phase cultures of the *E. coli* and *Vibrio* strain at a ratio of 10:1. Then 100 µl was spotted onto Luria-agar plates supplemented as described below and incubated for 6 h at 30 °C. The bacteria were scraped from the agar, serially diluted and transconjugants selected by growth on agar plates containing rifampicin and carbenicillin at the concentrations given below.

Media and growth conditions. *Vibrio* sp. 60, with or without plasmids, was inoculated from frozen stocks [kept at -70 °C in Luria broth containing 1.5% (w/v) NaCl and 12.5% (v/v) glycerol] onto 1.5% (w/v) Bacto-agar plates containing Luria broth supplemented with NaCl to a final concentration of 1.5% and appropriate antibiotics, rifampicin (50 µg ml⁻¹) and carbenicillin (2 mg ml⁻¹), and then incubated overnight at 30 °C. Cells were scraped from the plates and inoculated to

an OD₆₀₀ of 0.02–0.05 in flasks containing between 10 and 50 ml Luria broth supplemented with NaCl. The cells were cultured at 30 °C on a rotary shaker, and growth was monitored by measuring the change in OD₆₀₀. For those strains containing plasmids pMMB68 or pMMB25 IPTG was added to a final concentration of 1.0 mM when the cells reached an OD₆₀₀ of approximately 0.2 in order to induce the expression of EtxB or catechol 2, 3-dioxygenase, respectively.

Cell fractionation. Samples of culture (usually 1.0 ml) were taken at different stages of growth. Media and cells were separated by centrifugation in an MSE microfuge (13000 r.p.m., 3 min, 20 °C), and the cells were resuspended in ice-cold 10 mM-Tris/HCl (pH 8.0) with 1% NaCl in a volume equivalent to the original sample volume. For determination of toxin concentration in the total cell fraction, the cells were firstly disrupted by sonication for 1 min (50% duty cycle) at 4 °C using an Ultrasonics model W-375 sonicator. For isolation of periplasmic fractions, the cells were washed once in ice-cold 10 mM-Tris/HCl (pH 8.0) with 1% NaCl, and either subjected to osmotic shock as described by Ichige *et al.* (1988) or treated with 100 units polymyxin-B ml⁻¹ for 15 min at 4 °C. The polymyxin-B-treated cells were separated from the released periplasmic contents by centrifugation at 4 °C in an Eppendorf centrifuge and the cell pellet was resuspended in 10 mM-Tris/HCl (pH 8.0), frozen at -20 °C, thawed on ice and sonicated for 10 s as above. The various cell fractions were stored at -20 °C, prior to further analysis.

Toxin and enzyme assays. The concentration of EtxB in media and cell fractions was determined by a GM1-ganglioside ELISA (GM1-ELISA), as described by Svennerholm & Holmgren (1978). β-Lactamase activity was assayed by measuring the hydrolysis of nitrocefin (Difco) and catechol 2,3-dioxygenase activity was monitored by measuring the oxidative cleavage of catechol, as described previously (Hirst & Holmgren, 1987a).

Gel electrophoresis. SDS-PAGE, w/v, (12.5% acrylamide) was performed on a Biorad Protean II system using buffer conditions recommended by the manufacturer.

Results

Expression of EtxB in *Vibrio* sp. 60

Previously it was found that the secretory mechanism required for the efficient and selective secretion of cholera-like enterotoxins into the extracellular medium is present in all pathogenic members of the *Vibrionaceae*, including both aeromonads and vibrios (Hirst & Holmgren, 1987a; Hirst & Leece, 1991; T. R. Hirst, T. Amin, M. Sandkvist, & M. Bagdasarian, unpublished observations). To examine whether the non-pathogenic marine micro-organism *Vibrio* sp. 60 possessed a similar toxin secretory machinery capable of secreting cholera-like enterotoxins into the medium, a plasmid encoding the B subunit of *E. coli* heat-labile enterotoxin (EtxB) was transferred into the strain and the expression and location of EtxB was analysed. To achieve this, a broad host-range plasmid, pMMB68, was used which carries the gene encoding EtxB under the control of an inducible

Table 1. Heterologous expression of EtxB in *Vibrio* sp. 60

Strain	IPTG induction*	Concn of EtxB ($\mu\text{g ml}^{-1}$)†					
		α -CT Media Cells		α -EtxB Media Cells		α -118/87 Media Cells	
<i>Vibrio</i> sp. 60	—	0	0	0	0	0	0
<i>Vibrio</i> sp. 60(pMMB68)	—	0.11	0.01	0.1	0.003	0.1	0.01
<i>Vibrio</i> sp. 60(pMMB68)	+	12.6	3.4	15.8	5.8	16.2	7.2

* *Vibrio* sp. 60 harbouring pMMB68 was grown in the absence (—) or presence (+) of 1 mM-IPTG.

† Determined by GM1-ELISA using polyclonal rabbit anti-cholera toxin (α -CT), polyclonal mouse anti-EtxB (α -EtxB) and mouse monoclonal antibody (α -118/87).

tac promoter (Sandkvist *et al.* 1987). This plasmid was mobilized into a rifampicin-resistant derivative of *Vibrio* sp. 60, as described. Expression and secretion of EtxB was investigated by growing the cells in Luria broth supplemented with NaCl to an OD₆₀₀ of approximately 1.0, followed by the sampling of media and cells for analysis of the B subunit concentration by GM1-ELISA (Table 1). As a control, the wild-type strain which lacked pMMB68 was also tested in this assay. A variety of toxin-specific antibodies were used in the ELISA, including polyclonal sera raised against cholera toxin and EtxB, and a monoclonal antibody against EtxB. The assays showed that the wild-type strain did not produce any detectable cross-reacting cholera-like enterotoxin (Table 1). In contrast, *Vibrio* sp. 60 harbouring pMMB68 produced high-levels of EtxB whose expression was dependent on the addition of IPTG to the growth medium. At least 70% of the EtxB that was produced was released into the medium.

Comparison of EtxB expression in the wild-type and a sec mutant of *Vibrio* sp. 60

An *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)-induced mutation in *Vibrio* sp. 60 with a pleiotropic defect in the secretion of extracellular proteins was isolated by Ichige *et al.* (1988). To establish the extent of the pleiotropic nature of the *sec* phenotype of this mutant, pMMB68 was mobilized into a rifampicin-resistant derivative of strain MVT1192. The expression and cellular distribution of EtxB in this strain was compared with that of the wild-type strain harbouring pMMB68 (Fig. 1). The growth rates and final optical densities of the two strains were similar, but the distribution of EtxB was markedly different. In the *sec* strain (Fig. 1b) the majority of the B subunit was located in the cells, with only approximately 5% being released into the medium during exponential-phase growth. This showed that the *sec* phenotype exhibited by this mutant also blocks release of EtxB.

Fig. 1. Expression of EtxB in rifampicin-resistant derivatives of marine *Vibrio* sp. 60 (a) and a mutant (MVT1192) that is defective in protein secretion (b). Bacteria harbouring plasmid pMMB68 were cultured in Luria broth supplemented with NaCl and 1 mM-IPTG, and growth was monitored by measuring OD₆₀₀ (○). At the time points indicated the concentration of EtxB in the medium (□) and in a fraction of sonicated cells (■) was determined by GM1-ELISA.

Samples of media from exponentially growing cultures of the wild-type and the *sec* strain were analysed by SDS-PAGE (Fig. 2). The medium from the wild-type strain lacking pMMB68 contained several major extracellular proteins, whereas the medium from the same strain harbouring pMMB68 contained one additional major protein. This protein had an electrophoretic mobility identical to that of purified EtxB (Fig. 2, compare lanes 1 and 3). *Vibrio* sp. 60 MVT1192(pMMB68) failed to

Fig. 2. Medium from exponentially growing cultures of *Vibrio* sp. 60 and *Vibrio* sp. 60 (MVT1192) with or without plasmid pMMB68. Strains were inoculated into Luria broth and IPTG was added when the OD₆₀₀ reached 0.2. Three hours after IPTG induction, the cells and media were separated by centrifugation and 50 µl of medium from *Vibrio* sp. 60 (lane 1), *Vibrio* sp. 60 (MVT1192) (lane 2), *Vibrio* sp. 60 (pMMB68) (lane 3) and *Vibrio* sp. 60 (MVT1192)(pMMB68) (lane 4) was analysed by SDS-PAGE (12.5%). The molecular mass markers used were (kDa): rabbit muscle phosphorylase b, 97.4; bovine serum albumin, 66.2; hen egg white ovalbumin, 42.7; bovine carbonic anhydrase, 31.0; soybean trypsin inhibitor, 21.5; hen egg white lysozyme, 14.4; and the B subunit of heat-labile enterotoxin, 11.7. The arrow indicates the migration position of the B subunit of heat-labile enterotoxin (EtxB).

release sufficient of this protein into the medium for it to be detected by Coomassie Blue staining (Fig. 2, lane 4). Analysis of the same samples by SDS-PAGE without prior heating to 100 °C, resulted in the putative EtxB protein migrating as a discrete oligomer with an

apparent molecular mass identical to that of purified EtxB pentamers (data not shown).

To confirm that the integrity of the outer membrane of *Vibrio* sp. 60 had remained intact during the release of EtxB, the distribution in the medium and cells of the periplasmic enzyme, β -lactamase, was also determined (Table 2). Ninety-seven percent of the β -lactamase activity was found to be associated with the cells during the release of EtxB by *Vibrio* sp. 60(pMMB68) (Table 2). It was concluded that release of EtxB by *Vibrio* sp. 60 occurs via a selective and efficient secretory mechanism and not by the non-specific release of periplasmic proteins.

Location of the cell-associated EtxB in the *sec* mutant

To determine the location of the cell-associated EtxB, two fractionation methods for release of periplasmic proteins were evaluated. A plasmid, pMMB25 (Hirst & Holmgren, 1987a) which encodes the periplasmic marker enzyme β -lactamase and the cytoplasmic marker enzyme catechol 2,3-dioxygenase, was introduced into the *Vibrio* sp. 60 wild-type and the *sec* mutant. Fractionation by osmotic shock using conditions identical to those reported by Ichige *et al.* (1988) for the characterization of *Vibrio* sp. 60 was found to result in the release of only 54–56% of the periplasmic marker enzyme β -lactamase. Therefore, an alternative method was used for the release of periplasmic proteins, using polymyxin B (Hirst & Holmgren, 1987a). The treatment of cells with 50–200 units polymyxin B ml⁻¹ for 15 min at 4 °C was sufficient to release more than 80% of the periplasmic β -lactamase, with release of less than 0.02% of catechol 2,3-dioxygenase. Fractionation of *Vibrio* sp. 60 (MVT1192)(pMMB68) with 100 units polymyxin B ml⁻¹ resulted in the release of 87% of the cell-associated EtxB from the *sec* mutant (Table 3). It was concluded that EtxB accumulates within the periplasm of the *sec* mutant and that the pleiotropic defect in secretion prevents the translocation of the toxin subunits from the periplasm across the vibrio outer membrane.

Table 2. Distribution of β -lactamase and EtxB in the wild-type and a *sec* mutant of *Vibrio* sp. 60

Strain	β -Lactamase activity (U ml ⁻¹)*		EtxB concn (µg ml ⁻¹)†	
	Medium‡ (Percentage of total)	Cells‡ (Percentage of total)	Medium‡ (Percentage of total)	Cells‡ (Percentage of total)
<i>Vibrio</i> sp. 60(pMMB68)	0.011 (3)	0.325 (97)	14.9 (96)	0.70 (4)
<i>Vibrio</i> sp. 60 (MVT1192) (pMMB68)	0.038 (5)	0.758 (95)	0.26 (2)	16.8 (98)

* Determined by hydrolysis of nitrocefin.

† Determined by GM1-ELISA using a mouse monoclonal antibody (α -118/87).

‡ Culture (1 ml) was centrifuged to separate the cells from the growth medium. The cells were then resuspended in an equal volume of fresh medium and lysed by sonication.

Table 3. Cellular location of EtxB in the wild-type and a *sec* mutant of *Vibrio* sp. 60

Strain	Concn of EtxB ($\mu\text{g ml}^{-1}$)*		
	Medium	Periplasm	Extracted cells
<i>Vibrio</i> sp. 60(pMMB68)	15.3	2.4	1.18
<i>Vibrio</i> sp. 60 (MVT1192) (pMMB68)	0.88	15.4	2.35

* Determined by GM1-ELISA using mouse monoclonal antibody (α -118/87).

Discussion

In this paper it is shown that the B subunit of *E. coli* heat-labile enterotoxin is efficiently and selectively secreted by *Vibrio* sp. 60 into the extracellular growth medium. Previous studies on the expression of the A and B subunits of *E. coli* enterotoxin or EtxB alone in *V. cholerae* had revealed that toxin secretion involves two translocation events in which the toxin subunits cross the cytoplasmic membrane as separate precursor polypeptides, followed by the assembly of the mature subunits into an oligomeric complex, and then the secretion of the holotoxin through the outer membrane (Neill *et al.*, 1983a; Hirst *et al.*, 1984a, b; Hirst & Holmgren, 1987a, b; Sandkvist *et al.*, 1987; Hirst, 1991). In contrast, expression of cholera toxin or the related *E. coli* enterotoxins in *E. coli* or in other members of the *Enterobacteriaceae* was found to result in the accumulation of assembled toxins within the periplasm (Palva *et al.*, 1981; Neill *et al.*, 1983b; Hirst *et al.*, 1984b; Hofstra & Witholt, 1984, 1985; Hardy *et al.*, 1988; Hirst & Leece, 1991; Sixma *et al.*, 1991). The similarity in the efficiency and selectivity of EtxB secretion by *Vibrio* sp. 60 and *V. cholerae* suggests that the pathway and mechanism of toxin secretion in the two organisms is likely to be identical.

An analysis of media samples from the wild-type *Vibrio* sp. 60 strain not harbouring the B subunit-encoding plasmid showed that this organism doesn't produce a detectable cholera-like enterotoxin. This was not too surprising, since *Vibrio anguillarum* is a marine micro-organism and a recognized non-pathogen (Janda *et al.*, 1988). What is perhaps more intriguing is the reason why *Vibrio* sp. 60 should possess a secretory machinery that translocates cholera-like toxins through its envelope into the medium, when it does not normally appear to produce such toxins.

One possible explanation is that all vibrios possess a pleiotropic secretory machinery which is responsible for translocating many different proteins, including oligomeric toxins, across their outer membranes into the extracellular medium. Consistent with this hypothesis is our finding that a *sec* mutant of *Vibrio* sp. 60 which is

pleiotropically-defective in the secretion of several extracellular proteins, also fails to secrete EtxB into the medium. Similarly, the heterologous expression of aerolysin from *Aeromonas hydrophila* in *Vibrio* sp. 60 resulted in aerolysin secretion from the wild-type but not from *sec* strains (Wong *et al.*, 1990). Thus, the mutation which defines the *sec* phenotype in MVT1192 would appear to block the secretion of many different proteins and to cause their accumulation within the periplasm. If such a pleiotropic mechanism does indeed exist it would have to be able to discriminate between resident periplasmic proteins and those passing through the periplasm *en route* to the outer membrane translocation machinery. This would only be conceivable if either all secreted proteins possessed a common structural feature or targeting motif that permitted similar interaction with the outer membrane and/or entry into the secretory machinery, or each secreted protein interacted with a different factor or 'gatepost' at the site of entry into a common secretory machinery. As yet, no universal targeting motif for translocation across the outer membrane has been identified; and moreover such a motif would seem unlikely given the almost certain differences in structure between assembled EtxB pentamers and those of the protease, amylase, DNAase, soluble hemagglutinin and aerolysin that are also secreted from *Vibrio* sp. 60.

An alternative explanation would be that there is more than one type of secretory machinery, each determining the secretion of a different protein or sub-set of proteins across the outer membrane. In this case, the mutation in MVT1192 must effect the functioning of all of the different translocatory mechanisms in the same way. This could occur if the process of protein translocation across the outer membrane relies on a feature or property of that membrane for efficient translocation to happen. For example, it is conceivable that the constituent lipids, or the integrity of the membrane, or a putative membrane potential (Wong & Buckley, 1989) could be affected in MVT1192 which causes the pleiotropic inhibition of all outer membrane translocatory mechanisms. Indeed, Ichige *et al.*, (1988) reported that all *sec* mutants of *Vibrio* sp. 60 were difficult to transduce with

phage As3, suggesting a change in cell surface structure. It remains to be established whether the mutations in *Vibrio* sp. 60 define genes which encode a pleiotropic secretory apparatus, or the integrity and normal functioning of several membrane processes, including secretion of extracellular proteins.

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