Cloning and characterization of a novel haemolysin in *Vibrio cholerae* O1 that does not directly contribute to the virulence of the organism

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A previously undescribed haemolysin, distinct from the major *Vibrio cholerae* O1 El Tor haemolysin, HlyA, was cloned from the O1 classical biotype strain Z17561. This novel haemolysin showed 71.5% overall similarity to the δ-thermostable direct haemolysin of *Vibrio parahaemolyticus*, and so it has been termed *V. cholerae* δ-thermostable haemolysin (Vc-δTH, encoded by the dth gene). An ORF found immediately downstream, which appears to be transcriptionally and translationally linked to dth, displayed strong homology to the family of acyl-CoA synthetases. When expressed from an inducible promoter in *Escherichia coli*, Vc-δTH was shown to be a 22.8 kDa protein active on sheep red blood cells. Co-expression of acs with dth had no effect on the haemolytic activity or cytoplasmic localization of Vc-δTH. A *V. cholerae* Z17561 dth::KmR mutant showed unaltered behaviour in the infant mouse cholera model.

**Keywords:** enteric disease, virulence factor acyl-CoA synthetase, thermostable

**INTRODUCTION**

*Vibrio parahaemolyticus* is one of the causative agents of food poisoning, and although not all strains are considered to be pathogenic, it is the major cause of acute gastroenteritis associated with seafood consumption (Janda *et al.*, 1988). *V. parahaemolyticus* produces a thermostable direct haemolysin (TDH) which is responsible for the haemolytic activity displayed on special blood agar (Wagatsuma agar), termed the Kanagawa phenomenon (KP) (Honda *et al.*, 1991; Iida & Yamamoto, 1990). The KP is usually only observed in strains isolated from clinical cases, whereas most environmental strains are non-haemolytic: consequently, KP has been conveniently used as a virulence marker for *V. parahaemolyticus* (Janda *et al.*, 1988; Miyamoto *et al.*, 1969; Nishibushi *et al.*, 1992; Nishibushi & Kaper, 1995).

TDH is a homodimer protein with a molecular mass of 46 kDa, each monomer consisting of 165 amino acids (Takeda *et al.*, 1978). It is a pore-forming toxin, able to lyse erythrocytes from a number of mammalian species (Honda & Iida, 1993). In addition to its haemolytic activity, TDH displays cardiotoxicity, mouse lethality, enterotoxicity and cytotoxicity to cultured cells (Takeda, 1983).

Other diarrhoea-causing *Vibrio* species also produce haemolysins similar to TDH, including non-O1 *Vibrio cholerae*, *Vibrio mimicus* and *Vibrio hollisae*. The coding sequences of the corresponding genes have greater than 93% homology to TDH and have therefore been included in the *tdh* family. Interestingly, KP-negative strains of *V. parahaemolyticus* still capable of causing gastroenteritis have now been found to produce a TDH-related haemolysin (TRH) (Honda *et al.*, 1988). TDH and TRH share common epitopes, with approximately 70% sequence identity (Nishibuchi *et al.*, 1989). However, unlike the *tdh* genes, significant nucleotide
difference exists within the trb family, with two subgroups, trb1 and trb2, sharing 84% sequence identity. A thermolabile haemolysin (TLH) has also been found in *V. parahaemolyticus*, which, unlike the TDH and TRH pore-forming toxins, displays phospholipase activity (Taniguchi et al., 1985, 1986).

More recently, another thermostable haemolysin (δ-VPH) has been identified, and found to be present in all strains of *V. parahaemolyticus* tested (Taniguchi et al., 1990). The nucleotide and predicted amino acid sequence differ from those of the tdh, trb and tlb genes, or any other haemolysins of *V. parahaemolyticus*. In this study, we report the cloning and sequencing of the gene (*dbh*) encoding a thermostable haemolysin from the *V. cholerae* O1 strain Z1756, this haemolysin shows homology to the δ-VPH of *V. parahaemolyticus*. The genetic organization of the *dbh* region has been examined, and the contribution of this haemolysin to pathogenicity has been evaluated by construction of a mutant and assessment in vivo.

### METHODS

**Bacterial strains, plasmids and media.** *V. cholerae* O1 strain Z17561 of the Classical biotype was used as the source of chromosomal DNA for cloning and introducing a chromosomal mutation into *dbh*. Other *V. cholerae* O1 strains used were S69B (classical, Inaba), O17 (El Tor, Ogawa), H1 (El Tor, Ogawa) and O17 bbyA::Km, an El Tor haemolysin-defective mutant described elsewhere (Alm et al., 1991). The *V. cholerae* O139 serotype strains used were A1-1837 and Arg3. The *V. parahaemolyticus* strains used were NCTC 10884 (Kanagawa positive) and NCTC 10885 (Kanagawa negative).

*Escherichia coli* K-12 and *V. cholerae* strains were grown in nutrient broth (Oxoid) at 37 °C with shaking. Plasmids used in this study were pBluescript SK (Stratagene), pGEM-T and pGEM3Zf (Promega), pJLA503 (Schauer et al., 1987), pT7-3 (Tabor & Richardson, 1985) and pCDV442 (Donnenberg & Kaper, 1991). *E. coli* DH5az was used for general plasmid transformations, and strain S17-1pir was used as the permissive donor strain, for the suicide vector pCDV442. X-Gal and IPTG were both used at a final concentration of 40 µg ml⁻¹. Antibiotics were supplemented at the following concentrations: ampicillin, 50 µg ml⁻¹; and kanamycin, 50 µg ml⁻¹. Blood agar was prepared by adding 5% packed washed sheep, rabbit or human erythrocytes to nutrient agar.

**Haemolysis assay.** Haemolysis assays were performed using either *E. coli* culture supernatants or whole-cell lysates. An aliquot of the sample to be examined was mixed with 100 µl of a 8% suspension of washed sheep red blood cells and incubated at 37 °C for 15 min unless otherwise specified. Aliquots were taken at various time points and pelletted in a microcentrifuge for 45 s. Haemolytic activity (haemoglobin release) was assessed by measurement of A₅₄₀ after appropriate dilution. Carbohydrates for the haemolysis inhibition assays were used at a final concentration of 30 mM; dextran 4 was from Serva Feinbiochemia.

**Recombinant DNA techniques and nucleotide sequence analysis.** Restriction enzyme digests, ligation of DNA fragments, agarose gel electrophoresis, transformation of *E. coli* and Southern blot analysis were performed as described by Sambrook et al. (1989). Plasmid DNA was prepared by alkaline lysis (Sambrook et al., 1989), and genomic DNA was prepared as described by Manning et al. (1986). PCR amplification was carried out using standard protocols with the oligonucleotides described in the text. Taq polymerase (Hoffmann La-Roche) was used in all reactions. All PCR products used were sequenced to confirm fidelity of the polymerase.

Oligodeoxynucleotide primers used were: #2613 (5'-ACCCGTATTATAGCCCTGCT-3'), #2614 (5'-TATGGATAAGTTCAGCCG-3'), #2621 (5'-CTTGGACCAACCCGACAAG-3'), #2622 (5'-GCCAAATCACCAGACGACAGA-3'), #2646 (5'-TACGGTTACCTCTGCTGCT-3'), #2647 (5'-CCCGAGTTTACGGAAGAC-3'), #2656 (5'-ATATGCTTTCTTACCTGTC-3'), #2863 (5'-TTATGCAATTCGCGTCC-3'), #2864 (5'-GTATTTCGGACCGGCTTTG-3') and #2879 (5'-GTCACCAAGGCTGCTGACCAT-3').

DNA sequencing of pPM5118 was accomplished by the initial use of the universal primers M13F and M13R. Primer walking by designing oligo primers after downstream sequence was allowed the entire DNA fragment to be sequenced in both directions. Primers used in the forward direction were #2613, #2621, #2646 and #2656. Primers used for sequencing in the reverse direction were #2614, #2622, #2647 and #2657. Plasmid pPM5122, containing the downstream region of the *acss* ORF, was sequenced initially using the primers M13R and #2656, followed by #2860 and #2879 by primer walking.

**Library construction and screening.** A size-selective genomic library of *V. cholerae* strain O1 Z17561 was constructed in pBluescript SK using chromosomal DNA partially digested with *Sau3A1*. DNA fragments of approximately 2 kb were agarose gel purified and cloned into the BamHI site of the plasmid. Clones with insert DNA were identified by blue/white colour selection and prepared randomly for sequencing. DNA sequencing of pPM5118 was accomplished by the initial use of the universal primers M13F and M13R. Primer walking by designing oligo primers after downstream sequence was allowed the entire DNA fragment to be sequenced in both directions. Primers used in the forward direction were #2613, #2621, #2646 and #2656. Primers used for sequencing in the reverse direction were #2614, #2622, #2647 and #2657. Plasmid pPM5122, containing the downstream region of the *acss* ORF, was sequenced initially using the primers M13R and #2656, followed by #2860 and #2879 by primer walking.

**Chromosomal insertional mutagenesis.** To construct a *dbh* insertion mutant in *V. cholerae* Z17561, a 1.28 kb HincII Km⁸ cartridge was inserted into a unique NcoI restriction site of the ORF in pPM5118 after end-filling with Klenow. A SalI fragment containing the entire ORF with the Km⁸ cartridge was then isolated and ligated into the SalI restriction site of pCDV442 (Donnenberg & Kaper, 1991), producing pPM5121. Following transformation into S17-1pir, the plasmid construct was conjugated into *V. cholerae* Z17561 at a donor/recepent ratio of 1:10. The cells were centrifuged, resuspended in 0.3 ml nutrient broth and spread onto a 0.45 µm-pore-size Millipore filter laid onto a nutrient agar plate for 4 h at 37 °C. Km⁸ *V. cholerae* exconjugants arising from a single cross-over event were isolated on Vibrio selective TCBS Cholera Medium (Oxoid) containing kanamycin. A single colony was selected and grown overnight in nutrient broth in the presence of...
kanamycin and 6% sucrose. The presence on pCVD442 of the Bacillus subtilis sacB gene encoding levansucrase allows positive selection for loss of the integrated plasmid via a second recombination event, as the product of levansucrase (Sac B) is lethal to bacterial cells in the presence of sucrose; maintenance of the insertional inactivated gene is monitored by kanamycin resistance. Fifty colonies were then patched onto Ap plates to screen for loss of the plasmid. Colonies that were Km\(^b\) and Ap\(^b\) were presumed to be dth: Km\(^b\) and were confirmed by PCR and Southern blot analysis.

**Protein expression and cell fractionation.** For overexpression of proteins using the T7 polymerase/promoter system, plasmid pET17b (Novagen) containing the cloned gene of interest was transformed into *E. coli* DH5\(^{\alpha}\) with pGPEI-2, which contains the T7 RNA polymerase under control of the 2\(\alpha\)ts857 repressor (Tabor & Richardson, 1985). Plasmid pJLA503 containing the \(\lambda\) rightward promoter was also used by the method described by Schauder et al. (1987). Fractionation of *E. coli* K-12 cells into soluble fractions (cytoplasm and periplasm) and insoluble fractions (inner membrane and outer membrane) was carried out as described previously (Achtman et al., 1979). Samples were visualized by Coomassie blue staining following SDS-PAGE as described by Lugtenberg et al. (1975). All samples were boiled for 3 min in SDS loading buffer at 100 °C prior to loading. Protein marker standards used were from Pharmacia.

**Infant mouse work.** The *in vivo* consequences of introducing a dth: Km\(^b\) mutation into the *V. cholerae* Z17561 strain were assessed in the infant mouse cholera model. The virulence of the parent and of the isogenic mutant were compared by performing simultaneous (48 h) LD\(_{50}\) titrations in young Swiss mice (2\(\times\)4–2\(\times\)7g) as described elsewhere (Attridge & Rowley, 1983), using five animals per dose. A mixed-infection competition experiment was also performed. Mutant and wild-type strains were cultured separately and a mixed suspension prepared, such that each mouse would receive approximately 2 \(\times\) 10\(^8\) bacteria of each strain (approx. one LD\(_{50}\) dose of the parent strain). The precise ratio of parent:mutant bacteria was determined retrospectively, by plating aliquots of (a suitable dilution of) the inoculum on to nutrient and Km plates. After 23 h, the animals were killed and their small intestines excised, homogenized and plated on both media, to allow determination of the relative recoveries of parent and mutant bacteria. Studies in the infant mouse cholera model were performed with the approval of the Animal Ethics Committee of the University of Adelaide.

### RESULTS AND DISCUSSION

**Sequence analysis of the *V. cholerae* dth region**

A gene library of *V. cholerae* Z17561 chromosomal DNA was generated by partial *Smu*AI digestion and cloning into the plasmid vector pBluescriptSK. The random sequencing of inserts using the universal primers M13F and M13R identified one clone with homology to the \(\delta\)VPH of *V. parahaemolyticus* (Taniguchi et al., 1990). This particular clone was of interest to us as it contained a potential virulence factor not previously described for *V. cholerae* O1. The resulting plasmid, pPM5118, containing a 2.0 kb insert, was therefore fully sequenced in both directions, revealing an ORF encoding a protein with 50.5% identity and 71.5% overall similarity to the thermostable haemolysin (\(\delta\)-VPH) from *V. parahaemolyticus* (Taniguchi et al., 1990). We therefore termed this putative haemolysin gene *dth*, for *V. cholerae* delta thermostable haemolysin, and the corresponding protein was named Vc-\(\delta\)TH (Fig. 1).

Interestingly, the two haemolysins have two regions of near-complete identity, containing two conserved cysteine residues (see Fig. 1). Two cysteine residues form an intra-chain bond in the subunit of TDH (Tsunasawa et al., 1987) and are also conserved in the TRH subunit (Nishibuchi et al., 1989). While we do not at present have any evidence to show that Vc-\(\delta\)TH forms a homodimer, the presence of these highly conserved cysteine residues suggests that it may also exist as a two-subunit complex.

A putative ribosome-binding site (AAAGGAA) is present 6 bases upstream of the proposed initiation codon. Furthermore, an *E. coli* \(\sigma^{34}\) consensus –10 promoter sequence (TATATT) 47 bases further upstream of the start site was identified using the network promoter prediction program at the Berkeley Drosophila Genome Project web site (www.fruitfly.org). However, no –35 promoter consensus sequence is apparent; this may limit expression of this gene in *V. cholerae*, or indicate the requirement of an activator protein for full expression. In support of this finding, no haemolytic activity could be detected in *E. coli* harbouring pPM5118 on blood agar plates containing human, horse, sheep or chicken erythrocytes (incubation times of up to 48 h; data not shown). Moreover, no residual haemolytic activity could be detected in a *V. cholerae* mutant (O17 hlyA:: Km\(^b\)) in which the major haemolysin had been insertionally inactivated (Alm et al., 1991). While this suggests that the Vc-\(\delta\)TH homologue is either not expressed or very poorly expressed in *V. cholerae*, it may also reflect the fact that the necessary growth conditions required for its expression are not achieved *in vitro*.

The complete ORF *dth* encodes a predicted protein of 22.8 kDa but, in contrast to the TDH of *V. parahaemolyticus* (Myonsum et al., 1991), no signal peptide could be deduced from the predicted sequence. However, this is in agreement with the *V. parahaemolyticus* \(\delta\)-VPH, which also contains no typical features of a signal sequence at its amino-terminus (Taniguchi et al., 1990).

### Cloning of the *acs* gene

The partial ORF downstream of *dth* showed best overall homology to the bacterial fatty acyl-CoA synthetases (Kameda & Nunn, 1981), and we therefore termed this putative gene *acs* for *V. cholerae* acyl-CoA synthetase. Southern analysis was performed with a 400 bp PCR amplified probe, using the oligos \#2646 and \#2614, which bound at the end of the incomplete *acs* ORF (see Fig. 2). There are two *Pst*I sites upstream of this probe region immediately after the end of *dth* (Fig. 2). By probing with the \#2646/2614 PCR product on *Pst*I-digested chromosomal DNA, a 2.4 kb band was detected (data not shown). Approximately 1 kb of this fragment
Fig. 1. Comparison of the amino acid sequences (Altschul et al., 1990) of \textit{V. parahaemolyticus} \(\delta\)-VPH (Taniguchi et al., 1990) and \textit{V. cholerae} \text{Vc-}\(\delta\)-TH deduced from the nucleotide sequences of the \(\delta\)-thd and dth genes. Identical amino acids are indicated by an asterisk below the sequence, alignment gaps by hyphens (-), and conservative changes by a dot (.). Boxed areas show the regions of highest homology. Underlined characters correspond to the conserved cysteine residues and a Val, Lys, Arg (VKR) motif present in some cytolysins (Baba et al., 1992).

Fig. 2. Genetic map of the \textit{dth} region cloned from \textit{V. cholerae} Z17561. ORFs VCA1108 to VC1111 are labelled according to the nomenclature of Heidelberg et al. (2000). VCA1111 has homology to the \(\delta\)-thermostable haemolysin of \textit{V. parahaemolyticus} (Taniguchi et al., 1990). VCA1110 has homology to the acyl-CoA family of synthetases (Black et al., 1997). Horizontal arrows indicate primer binding positions used for generating DNA probes and PCR cloning. Plasmid pPM5118 represents the original \textit{Sau}3AI clone obtained from random screening of a partial \textit{Sau}3AI gene library of \textit{V. cholerae} Z17561. See text for further details.

represented the end of the original clone in pPM5118. Therefore it was assumed that the rest of the \textit{acs} ORF was present on this downstream fragment, representing a further 1–4 kb.

In order to clone this fragment, vector-assisted PCR rescue was used. \textit{PstI}-digested Z17561 chromosomal DNA was ligated to \textit{PstI}-digested and alkaline-phosphatase-treated vector pGEM3zf, followed by PCR. Oligo \#2656, which binds at the end of the incomplete \textit{acs} ORF and reads outwards of this ORF (see Fig. 2), was used in conjunction with M13 reverse primer, which binds to the plasmid pGEM3zf. An approximately 1–4 kb band was expected from this PCR product, based on the mapping of the downstream \textit{PstI} site by Southern analysis. A 1–5 kb PCR product was obtained and cloned into pGEM-T, resulting in plasmid pPM5122. The DNA sequence showed overlap with the previous \textit{acs} sequence and extended the ORF to completion, with an additional complete third ORF (no known function) and a partial fourth (see Fig. 2).

The complete \textit{acs} ORF showed strongest homology with the bacterial fatty acyl-CoA synthetases, including FadD (33% similarity) from the hyperthermophilic archaeon \textit{Aquifex aeolicus} (Deckert et al., 1998) and the putative long-chain fatty acid ligase (38% similarity) from \textit{Streptomyces coelicolor} (Redenbach et al., 1996). Most notable in this regard is a 25 amino acid consensus sequence, DGWLTGIDIGXWPAEKRKKK,
common to all bacterial fatty acyl-CoA synthetases (Black et al., 1992). Within this consensus sequence are 8 invariant and 13 highly conserved amino acid residues which represent the fatty acyl-CoA synthetase (FACS) signature motif (Black et al., 1997). The acs ORF contains 17 amino acids that are either identical or highly conserved to the FACS signature motif (³⁵⁴DEW-LATGDLATLDEQGFLCIVGRKK³⁶⁰). This motif is essential for catalytic activity, and is thus thought to comprise part of the fatty-acid-binding site within the enzyme (Black et al., 1997).

We compared our sequenced 3.4 kb chromosomal fragment containing dth and acs from V. cholerae Z17561 with that of the recently completed genome sequence of V. cholerae El Tor N16961 (Heidelberg et al., 2000). Complete identity was observed with these regions of DNA, suggesting that this area of the chromosome is conserved amongst V. cholerae O1 strains. The overall G+C content of dth and the downstream ORFs was 50.46 mol%, which is comparable to the overall G+C content of V. cholerae. The dth gene (VCA1111) is present as a single copy on the smaller of the two V. cholerae chromosomes (chromosome 2) in strain N17561, at co-ordinates 1066499 to 1065900 reading in the minus strand orientation (Heidelberg et al., 2000; www.tigr.org).

Southern analysis with a PCR-generated probe using the oligos #2647 and #2613 (see Fig. 2), which are internal dth-based sequences, confirmed that this gene is present in only one copy in strain Z17561 (data not shown). In order to see if the dth gene was present in other V. cholerae strains, several O1 and non-O1 strains were analysed by PCR amplification using primers #2647 and #2613. The expected 380 bp fragment was present in the V. cholerae O1 strains Z17561, O17, 569B and H1, and the O139 strain AI-1837, but absent in the O139 strain Arg3, a recent clinical isolate from Argentina (data not shown). In comparison, the tdh gene is present in nearly all V. parahaemolyticus strains isolated from clinical specimens, and is absent in most environmental isolates. It is also present in some strains of V. mimicus, V. cholerae non-O1 and all strains of V. hollisae (Nishibuchi et al., 1985). The tdh genes of V. parahaemolyticus are flanked by insertion sequences (although transposition of these genes has not been demonstrated) and have therefore been collectively termed insertion-like sequence elements (Terai et al., 1991). However, the potential mobility of tdh suggests that V. parahaemolyticus may have acquired this gene from another organism in the past. This is consistent with the lower G+C content of tdh genes (30 mol%) (Nishibuchi et al., 1990) compared to the average G+C content of Vibrio chromosomes of 50 mol%. In contrast, the δ-VPH appears to be present in all V. parahaemolyticus strains regardless of their source of isolation, as determined by DNA hybridization studies (Taniguchi et al., 1990). Interestingly, in these studies insufficient levels of homology existed to detect the dth in V. cholerae O1, using a V. parahaemolyticus δ-VPH probe by Southern analysis (Taniguchi et al., 1990). Although there is no indication that dth and the surrounding genes are located on insertion sequence-like elements, a more comprehensive study of V. cholerae O1 and non-O1 strains is required to determine the true distribution of these genes in this species.

Expression and cellular localization of Vc-δTH in E. coli

Analysis of the dth gene did not predict a consensus promoter, particularly at the −35 region, and we therefore initially cloned dth into the expression vector pJLA503 under the control of the λ right promoter (Schauer et al., 1987). An NdeI site was incorporated into the ATG start site of dth via PCR using the oligonucleotide primers #2822 (incorporating a 5′ NdeI site) and #2622 to generate a 940 bp fragment. Digestion of this PCR product with NdeI and EcoRI gave rise to a 620 bp fragment, due to the presence of an internal EcoRI site 18 bp downstream of the stop codon of dth (see Fig. 2). This product was cloned directly into pJLA503 to give plasmid pPM5123.

Induction of gene expression in bacteria carrying pPM5123 required incubation at 42 °C for 3 h with exponential-phase cells initially grown at 30 °C. The samples were then fractionated, and as can be seen in Fig. 3, both whole cells and the cytoplasmic fraction showed a protein band of approximately 23 kDa in the presence of pPM5123. This in agreement with the predicted protein size of 22.8 kDa. The overexpressed protein is located exclusively in the cytoplasm, due presumably either to the lack of a suitable export signal (as no signal peptide was identified at the N-terminus of this protein), or to the absence of necessary accessory export genes which are not normally present in the E. coli host. This may not reflect the true situation for dth in V. cholerae, and the cloning of other genes needed for its secretion may be required to study this process further in E. coli. Interestingly, the apparent extracellular production of δ-VPH cloned into E. coli has not been conclusively confirmed, and may in fact simply reflect cell lysis (Taniguchi et al., 1990).

Other Vibrio species produce haemolysins similar to the TDH of V. parahaemolyticus, including non-O1 V. cholerae, V. mimicus and V. hollisae. The coding sequences of these genes have greater than 93% homology to V. parahaemolyticus TDH and are immunologically indistinguishable when tested with V. parahaemolyticus TDH polyclonal antiserum (Yoh et al., 1991). V. parahaemolyticus strains also produce a TDH-related haemolysin (TRH) which has approximately 70% sequence identity to TDH; while sharing some epitopes, the haemolysins are not immunologically identical (Honda et al., 1988). We therefore tested Vc-δTH against both V. parahaemolyticus TDH and V. parahaemolyticus TRH polyclonal antiserum (a gift from T. Honda, Osaka University, Japan) to see if any common epitopes existed between these three haemolysins. Western blot analysis was performed, using a number of V. cholerae O1 strains along with the E. coli
V. cholerae strain harbouring pPM5123 (Fig. 4). The E. coli strain expressing Vc-δTH was used as a positive control as the conditions under which dth is expressed in V. cholerae have not been determined. However, neither anti-TDH nor anti-TRH sera (data not shown) detected Vc-δTH, suggesting that this represents a novel haemolysin.

As a highly expressed protein band was seen after induction of the λ promoter in E. coli carrying pPM5123, this strain was tested for haemolytic activity. Whole-cell lysates and supernatants were prepared after 3 h induction at 42 °C and incubated with sheep erythrocytes. Approximately 50% of the haemolytic activity was present in the whole-cell lysate, while the remainder resided in the supernatant fraction (Fig. 5a, b). Vc-δTH is localized to the cytoplasmic fraction in E. coli and therefore the majority of activity was expected to be present in the whole-cell lysates. While some extracellular leakage can occur due to loss of cell integrity as a result of protein overexpression, the high proportion of haemolytic activity displayed in the supernatant fraction was unexpected. However, this is not reflected in the levels of Vc-δTH protein detected in the cytoplasm versus below detectable limits in the supernatant (Fig. 3, lanes 3 and 7), and may in fact represent differences in the activated state of Vc-δTH. Whether this is a result of intracellular aggregation of Vc-δTH in an inactive form in the cytoplasm, or a post-translational activation such as proteolytic processing upon release from the cytoplasmic compartment remains to be determined.

Although dth under the control of the strong λ promoter in pJLA03 was able to display haemolytic activity (not shown), we hypothesized that the acyl-CoA synthetase gene which appears to be translationally linked to dth may also be required, for appropriate cellular localization of Vc-δTH, or enhancing haemolytic activity through post-translational modification. Fatty acyl-CoA synthetases catalyse the formation of fatty acyl-CoA from fatty acid, ATP and CoA, and represent bioactive compounds that are involved in a number of cellular processes, including protein transport, enzyme activation and protein acylation. The best-studied example of post-translational modification of a cytolytic protein by an acyl transferase is that of the E. coli haemolysin

\( \text{Fig. 3. Subcellular localization of Vc-δTH in E. coli containing plasmid pPM5123 (lanes 1–6), pJLA03 (7–12) or pT7-3 (13–18). Plasmids pPM5123 (expressing dth), pT7-3 (expressing β-lactamase) and pJLA03 (with no insert) were induced for protein expression by heat inactivation of the cits857 repressor. Cells were fractionated to yield the following: whole cells (1, 7 and 13), cytoplasm (2, 8 and 14), periplasm (3, 9 and 15), unlysed cells (4, 10 and 16), whole membranes (5, 11 and 17) and supernatant (6, 12 and 18). Samples were separated on a 15% SDS-polyacrylamide gel followed by Coomassie blue staining. Arrows A indicate Vc-δTH in the whole-cell and cytoplasmic fraction. Arrows B indicate the pT7-3-encoded β-lactamase in the whole-cell and periplasmic fraction. M, molecular mass markers.} \)

\( \text{Fig. 4. Western blot analysis of whole-cell and supernatant samples of V. cholerae, V. parahaemolyticus and E. coli expressing Vc-δTH: V. cholerae O17 El Tor (lanes 1, 5 and 9), V. cholerae Z17561 Classical (2, 6 and 10), E. coli/pPM5123 (3, 7 and 11) and V. parahaemolyticus NCTC 10884 Kanagawa positive (4, 8 and 12). Cells were grown in KP broth, which is known to induce tdh expression in V. parahaemolyticus via the toxRS operon (Lin et al., 1993), prior to loading on a 15% SDS-polyacrylamide gel. Lanes 1–4 contain whole-cell samples; lanes 5–12 contain supernatant samples. Lanes 9–12 are identical to lanes 5–8, except that the samples were concentrated 10-fold by trichloroacetic acid precipitation. The primary antibody used was anti-V. parahaemolyticus TDH. The arrows indicate V. parahaemolyticus TDH. M, molecular mass markers.} \)
lac pAF46 contained both genes cloned downstream of the pGEM-T Easy into both orientations. The plasmid mic fraction) of Vc-

HlyA (Stanley et al., 1998). The inactive pro-toxin pro-
HlyA is activated by amide linkage of fatty acids to two internal lysine residues, directed by the co-synthesized HlyC protein, an acyl transferase, with acyl carrier protein as the fatty acid donor (Stanley et al., 1998). A role for a fatty acyl synthetase, RpfB, in the production of a diffusible extracellular factor (DSF) has also been shown to be important in Xanthomonas campestris pathogenesis (Barber et al., 1997). We therefore wanted to test the activity of Vc-\(\delta\)TH cloned and expressed in the presence of \(\alpha\)cs.

In order to co-express both \(\delta\)th and \(\alpha\)cs on the same plasmid, the primers \#2822 and \#2860 were used to PCR-amplify the DNA as a single fragment from the V. cholerae chromosome. Oligo \#2822 bound at the start of the \(\delta\)th gene while \#2860 bound at the stop codon of the \(\alpha\)cs gene (see Fig. 2). This 2.5 kb product was cloned into pGEM-T Easy into both orientations. The plasmid pAF46 contained both genes cloned downstream of the lac promoter, while pAF47 represented the genes under T7 promoter control. No difference was seen in the haemolytic activity and cellular localization (cytoplasmic fraction) of Vc-\(\delta\)TH in these strains when compared to the \(\delta\)th-containing clone pPM5123 (data not shown). It cannot be ruled out entirely that the \(\alpha\)cs gene plays some role in Vc-\(\delta\)TH activation or its cellular localization. However, the absence of other genes which may be required for complete function makes this difficult to assess in E. coli. The apparent transcriptional coregulation of downstream ORFs VCA1109 through to VCA1104 makes them ideal candidates for further investigation (www.tigr.org). Interestingly, two of these ORFs, VCA1105 and VCA1104, have similarity to the regulatory and sensory components of bacterial transduction systems. An authentic frameshift is present in VCA1104, the sensor histidine kinase component, in V. cholerae strain N17561, resulting in a truncated and presumably inactive protein. The role of this two-component regulatory system in \(\delta\)th expression, and whether the same mutation exists in V. cholerae Z17561, remains to be determined.

Preliminary evidence that the Vc-\(\delta\)TH is a pore-forming toxin is suggested by the ability of dextran 4 to act as an osmoprotectant. Haemolysis was prevented by the prior addition of dextran 4, whereas sucrose and raffinose had a lesser inhibitory effect (Fig. 6). The mean hydrated diameters of sucrose, raffinose and dextran 4 are 0.9, 1.1 and 3.5 nm respectively. Dextran 4 was able to prevent lysis, while the carbohydrates sucrose and raffinose, with molecular masses lower than that of dextran 4, were unable to do so. While these studies are only preliminary, assuming that pores formed by Vc-\(\delta\)TH act as a molecular sieve, then the pore diameter is estimated to be less than 3.5 nm. Given the high levels of expression of \(\delta\)th achieved in E. coli by use of the strong \(\lambda\) promoter, we plan to purify this protein in future for a more extensive study of its pore-forming properties.

**Mutant construction**

To evaluate the pathogenic significance of the Vc-\(\delta\)TH, a mutant of V. cholerae Z17561 was constructed by insertional inactivation of \(\delta\)th. The gene was interrupted by the insertion of a Km\(^R\) cassette and the resulting construct recombined into the chromosome using a

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**Fig. 5.** Haemolytic activity of whole-cell lysates (■, ■) and supernatants (○, ■) of E. coli expressing Vc-\(\delta\)TH. The strains examined were E. coli DH5\(\alpha\)/pPM5118, encoding \(\delta\)th (○, ○), and E. coli DH5\(\alpha\)/pJLA503 (vector control) (■, ■). (a) Haemolytic activity over time; (b) total activity with increasing amounts of whole-cell lysate or supernatant.

**Fig. 6.** Inhibition by dextran 4 of the haemolytic activity of whole-cell lysates and supernatants of E. coli expressing Vc-\(\delta\)TH. ▲, E. coli/pJLA503 (vector control); △, E. coli/pPM5123 (encodes \(\delta\)th under control of the \(\lambda\) promoter); ■, ○, ■, E. coli/pPM5123 with addition of sucrose (△), raffinose (○) or dextran 4 (■) before the haemolysis assay.
suicide vector (see Methods). The mutant Z17561 dth::KmR was confirmed by PCR and Southern hybridization and then compared with wild-type in the infant mouse cholera model.

An initial competition experiment failed to detect any defect in the mutant’s potential to persist in vivo. Mice received a mixed inoculum of parent:mutant bacteria at an input ratio of 1:0:1; when the two variants were separately enumerated from intestinal homogenates prepared 23 h later the median output ratio was 1:9:1 (n = 7; range 1:8:1–36:6:1). To eliminate the possibility that any disadvantage imposed by the dth mutation might have been compensated by Vc-δTH produced by the competing parent organisms, virulence titrations were performed. When tested in parallel both strains had the same LD50 dose of 1.3 × 10^8 bacteria. Together these data argue against a role for Vc-δTH in V. cholerae O1 pathogenesis, at least in this model.

Concluding remarks

The Vc-δTH represents a novel haemolysin, previously undescribed in V. cholerae. It is distinct from the V. paradoxaeamlyoticus TDH and TRH virulence-related haemolysins, with similarity to a less studied δ-VPH from this species (Tang et al., 1990). Unlike V. paradoxaeamlyoticus TDH, which is the major virulence factor in food-borne gastroenteritis, no direct role could be shown for Vc-δTH in V. cholerae O1 pathogenesis, although its contribution to the disease process may be masked by the multitude of other virulence factors elaborated by this species. Expression of dth in V. cholerae has not been detected with the in vitro culture conditions used, possibly reflecting the absence of appropriate environmental cues. Interestingly, the level of TDH production by V. paradoxaeamlyoticus is modulated by cultural conditions (Karunasagar, 1981), and the cloned dth genes require an exogenous promoter present on the plasmid vector for their expression (Nishibuchi & Kaper, 1985). Tang et al. (1990) were also unable to demonstrate haemolytic activity in the K1-negative V. paradoxaeamlyoticus K6 strain, and concluded that as yet undefined culture conditions were required for expression of the δ-VPH gene. A homologue to the V. cholerae toxRS operon has been identified in V. paradoxaeamlyoticus and found to mediate environmental regulation of TDH (Lin et al., 1993). Whether dth expression is affected by ToxRS or another such regulator in V. cholerae remains to be determined.

REFERENCES


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