Molecular Characterisation of the Haemolysin (HlyA) and the Region Downstream of *hlyA* in *Vibrio cholerae* O1

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A thesis sumitted for the degree of Doctor of Philosophy.

March, 1998.

Abstract

The haemolysin (HlyA) of El Tor *Vibrio cholerae* O1 is a potent toxin with both enterotoxic and cytotolytic activities and is considered an alternate diarrhoegenic factor to the cholera toxin, for El Tor and non-O1 strains. Although originally termed "haemolysin" because of its ability to lyse erythrocytes, it is also capable of lysing a variety of mammalian cells in culture. Osmotic protection and artificial lipid bilayer assays show that HlyA forms moderately anion selective pores with a single-channel conductance of 350pS in 1M KCl and an estimated diameter of <1.6 nm. HlyA is predicted to consist of amphipathic β-sheets which may be involved in pore formation.

Stable C-terminal truncations of HlyA were constructed in order to define structural domains that relate to function. Deletions at the C-terminus of pro-HlyA exposed a proteolytically sensitive site approximately 15 kDa from the C-terminus. Deletions of only 12 amino acids expose this site and cleavage occurs at this site efficiently in both *E. coli* and *V. cholerae*. Cleavage at the proteolytically sensitive site, together with the cleavage at the N-terminus that is normally required for activation, produces a 50 kDa derivative of HlyA. This central 50 kDa region of pro-HlyA was able to form pores in sheep erythrocytes and cause lysis, and defines a minimum haemolytic domain, characterised thus far. HlyA is homologous to the VAH1, ASH1 and AHH1 haemolysins of *V. anguillarum*, *A.salmonicida* and *A. hydrophila*, respectively, with a high degree of homology within the 50 kDa region of HlyA. The positions of the cysteine residues are conserved within these haemolysins, and are all contained within the 50 kDa domain, which may reflect conservation of secondary structure. The region Gln-609 to Thr-598 located at the C-terminus of 50 kDa HlyA (15 kDa from the C-terminus of pro-HlyA) is essential for the production of secreted, active haemolysin. The C-terminal 15 kDa of HlyA is not essential for secretion, pore-formation (cytolytic activity) or cleavage and so its role remains unknown.

HlyA is secreted as an 80 kDa pro-toxin that requires the removal of the N-terminal 15 kDa pro-region to release the mature 65 kDa protein. The soluble haemagglutinin / protease (SHA) produced by *V. cholerae* was identified as at least one factor that is involved

with the activation of HlyA. SHA is known to activate other virulence determinants of *V. cholerae*, including the cholera enterotoxin. Activation of HlyA was shown not to occur simultaneously with its secretion, as activity continues to increase in cell-free culture supernatants.

The region downstream of *hlyAB* was sequenced and characterised and a triacylglyceride-lipase operon (*lipAB*) encoding a 33 kDa lipase (LipA), and a 32 kDa accessory protein (LipB), was identified. LipA was previously known as HlyC, however as the amino acid sequence is highly homologous to the triacylglyceride-specific lipase of *Pseudomonas* spp, *hlyC* was re-named *lipA*. LipA contains the highly conserved pentapeptide and catalytic triad amino acid regions of the catalytic site of other lipases. LipB is homologous to the accessory-lipase proteins (lipase-specific foldase) required by *Pseudomonas* and various other bacterial species for the production of mature active lipase, and consistent with this, both *lipA* and *lipB* are required to restore a lipase-deficient *lipA* null mutant of *V. cholerae*. The intergenic stop codon for *lipA* overlaps the ribosome binding site for *lipB*, and a stem-loop resembling a rho-independent terminator is present immediately downstream from *lipB*, suggesting *lipA* and *lipB* form a lipase operon in *V. cholerae*.

A putative metalloprotease gene (*prtV*) was identified downstream of *lipAB*, but is transcribed in the opposite direction, and is predicted to share the same putative transcriptional terminator with *lipAB*. PrtV is 102 kDa and is highly homologous to the immune-inhibitor A (InA) metalloprotease of *Bacillus thuringiensis* and zinc-binding and catalytic domains conserved among many metalloproteases is present in PrtV.

The genetic organization of this region suggests it is possibly part of a pathogenicity island, encoding products capable of damaging host cells, or involved in nutrient acquisition by *V. cholerae*. However, neither *lipA* nor *prtV* null mutants were attenuated in the infant mouse model, nor did they exhibit reduced colonization potential when compared with wild-type in competition experiments.

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