Inactivation of two haemolytic toxin genes in *Aeromonas hydrophila* attenuates virulence in a suckling mouse model

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The contribution of two unrelated *Aeromonas hydrophila* β -haemolytic toxins to virulence was assessed in a suckling mouse model. The first haemolysin gene, isolated from an *A. hydrophila* A6 cosmid bank, encoded a potential gene product of 621 amino acids and a predicted molecular size of 69·0 kDa. The inferred amino acid sequence showed 89% identity to the AHH1 haemolysin of *A. hydrophila* ATCC 7966, and 51% identity to the HlyA haemolysin of *Vibrio cholerae* El Tor strain O17. The second haemolysin gene (designated *aerA*), which encodes aerolysin, a pore-forming toxin, was partially cloned by PCR for the purpose of mutant construction. This PCR product was a 1040 bp fragment from the C-terminal region of *aerA*. It is proposed that the 69·0 kDa *V. cholerae*-HlyA-like haemolysin gene be termed *hlyA* to contrast with the *aerA* terminology for the aerolysin. A suicide vector was used to inactivate both the *hlyA* and *aerA* genes in *A. hydrophila* A6. When assessed in the suckling mouse model, only the *hlyA aerA* double mutant showed a statistically significant reduction in virulence – a 20-fold change in LD₅₀ (Scheffe test, $P < 0.05$). Cytotoxicity to buffalo green monkey kidney cell monolayers and haemolysis on horse blood agar were eliminated only in the *hlyA aerA* double mutants. This is the first report of cloning and mutagenesis of two unrelated haemolytic toxin genes in the same strain of a mesophilic aeromonad. For *A. hydrophila*, a two-toxin model provides a more complete explanation of virulence.

Keywords: *Aeromonas hydrophila*, aerolysin, haemolysin, suckling mouse model, virulence

INTRODUCTION

The role of toxins as virulence factors in disease resulting from infection of the gut by Gram-negative organisms is well accepted. *Aeromonas hydrophila* is a ubiquitous Gram-negative organism of the aquatic environment (Okpokwasili, 1991; Alonso *et al.*, 1994; Papapetropoulou & Rodopoulou, 1994; Rhodes &

Kator, 1994; Sugita *et al.*, 1995) which has been implicated as a causative agent of human diarrhoea (Burke *et al.*, 1983; Agger *et al.*, 1985; Deodhar *et al.*, 1991; Delamorenna *et al.*, 1993; Ogunsanya *et al.*, 1994; Rautelin *et al.*, 1995; Utsalo *et al.*, 1995). For aeromonads, the use of different assays for the detection of toxins has resulted in complex and confusing nomenclature. The majority of *A. hydrophila* diarrhoeal isolates are β -haemolytic and produce cytotoxins. Two cloned and sequenced haemolytic toxin genes of *A. hydrophila* are those encoding AHH1 haemolysin (Hirono & Aoki, 1991) and aerolysin (Howard *et al.*, 1987). These haemolytic toxins have only 18% homology and are distinct (Hirono & Aoki, 1991).

The AHH1 haemolysin consists of 577 amino acid residues with a molecular mass of 63·7 kDa. The gene

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Abbreviation: BGMK, buffalo green monkey kidney.

The GenBank accession number for the 2265 kb DNA fragment encoding *hlyA* on pCWH3 is U81555.

Table 1. Strains and plasmids used or constructed in this study

Strain/plasmid	Relevant characteristics	Source/reference
<i>A. hydrophila</i> strains		
ATCC 7966	Wild-type; Ap ^r	Hirono & Aoki (1991)
Ah65	Wild-type; Ap ^r	Howard <i>et al.</i> (1987)
A6	Wild-type; Ap ^r	Atkinson & Trust (1980)
A6H	<i>hlyA</i> <i>EagI</i> deletion mutation	This study
A6A	<i>aerA</i> Km ^r insertion mutant	This study
A6HA	<i>hlyA aerA</i> mutant	This study
Plasmids		
pGEM-T	Ap ^r cloning vector	Promega
pUC18K	pUC18 with the non-polar <i>aphA</i> -3 Km ^r resistance cassette	Menard <i>et al.</i> (1993)
pCACTUS2	Suicide Cm ^r cloning vector, contains <i>sacB</i> and has a temperature-sensitive origin of replication	C. Clark and others (unpublished)
pCWH1	pHC79 carrying an approximately 40 kb fragment of <i>A. hydrophila</i> A6 genomic DNA	This study
pCWH2	<i>SphI</i> stepwise deletion of pCWH1	This study
pCWH3	<i>Clal</i> stepwise deletion of pCWH2	This study
pCWH4	pCWH3 with an 857 bp <i>EagI</i> fragment of <i>hlyA</i> deleted	This study
pCWH5	pCACTUS2 carrying the <i>SmaI</i> fragment of pCWH4 containing the deletion mutation	This study
pCWA1	pGEM-T carrying the 1040 bp PCR product derived from <i>aerA</i>	This study
pCWA2	pCWA1 carrying a Km ^r gene in the <i>SmaI</i> site	This study
pCWA3	pCACTUS2 carrying the <i>BamHI</i> fragment of pCWA2	This study

encoding this haemolysin was cloned and sequenced from *A. hydrophila* ATCC 7966 (Hirono & Aoki, 1991). The gene encoding a similar haemolysin, designated ASH4, was cloned from *Aeromonas salmonicida* 17-2 by Hirono & Aoki (1993). The ASH4 haemolysin gene encodes a 578 amino acid polypeptide with a molecular mass of 64.4 kDa which was 84% homologous to the AHH1 haemolysin.

Aerolysin consists of 463 amino acid residues with a molecular mass of 53.8 kDa. The gene encoding aerolysin was originally cloned and sequenced from *A. hydrophila* Ah65, a fish isolate (Howard *et al.*, 1987). X-ray crystallography studies showed that the haemolytic property of this toxin was mediated by pore formation in the erythrocyte cell membranes when seven of the aerolysin peptides come together to form a barrel structure (Parker *et al.*, 1994, 1996). In addition to its cytolytic activity to red cells and various tissue cell lines, aerolysin possesses enterotoxic activity (Rose *et al.*, 1989).

A. hydrophila A6, a diarrhoeal isolate, is lethal in suckling mice when administered by gavage (Wong *et al.*, 1996). The data presented in this paper show that strain A6 produces a *V. cholerae*-HlyA-like haemolysin in addition to aerolysin and it is the combined effect of both haemolytic toxins that causes the β -haemolysis on horse blood plates and the cytotoxicity to buffalo green monkey kidney (BGMK) cell lines, and that contributes to virulence in the suckling mouse assay.

METHODS

Bacterial strains and plasmids. Bacterial isolates and plasmids obtained or constructed in this study are listed in Table 1. Bacteria were cultivated in Luria broth or on Columbia agar. When required, defibrinated horse blood (5%) was added to the Columbia agar (HBA). Antibiotics were added to broth and solid media as described previously (Wong *et al.*, 1997). Minimal M9 medium (Miller, 1972) was supplemented with 2 μ g vitamin B₁ ml⁻¹, 2% glucose and/or 6% sucrose for the selection of resolved plasmids during allelic exchange in *A. hydrophila* A6.

Molecular methods. The preparation of chromosomal and plasmid DNA, construction of an *A. hydrophila* A6 cosmid bank, recombinant DNA techniques, and nucleotide sequencing and analysis have been described elsewhere (Wong *et al.*, 1997). Recombinant DNA clones containing the haemolysin gene of *A. hydrophila* were screened for β -haemolysis on HBA supplemented with ampicillin. The protocol for the amplification of the *aerA* and *hlyA* genes from *A. hydrophila* isolates was adapted from Wong *et al.* (1997).

Allelic exchange. The method of allelic exchange for the *aerA* and/or the *hlyA* genes in *A. hydrophila* was performed using the suicide vector pCACTUS2 and has been described previously (Wong *et al.*, 1997).

Suckling mouse model. Preparation of feeding inocula, infection of suckling mice and LD₅₀ determinations with *A. hydrophila* A6 and its mutants and the determination of accumulation of fluid in the gut (FA ratio) were followed according to the method of Wong *et al.* (1996). The FA ratio was expressed as the weight of the gut (mg) over the weight of the remaining carcass (g). The arbitrary cut-off value for

positive FA ratio is 80 mg g⁻¹. An FA ratio of 49 ± 4 mg g⁻¹ was considered as negative for accumulation of fluid in the intestine according to the criteria of Wong *et al.* (1996).

Statistical analysis. The one-way analysis of variance (ANOVA) and the Scheffe test were performed using the BMDP7D statistical software (BMDP Statistical Software, CA, USA).

Assay for cytotoxicity. The assay for cytotoxicity was performed with modifications from Wong *et al.* (1996). Briefly, 100 µl serial doubling dilutions (2–2048) of filter-sterilized culture supernatants of overnight bacterial cultures were added to a BG MK cell monolayer and incubated for 18 h at 37 °C in a humidified chamber. Cytotoxic effects on the monolayer were quantified using the crystal violet staining method of Matthews & Neale (1987).

Electrophoresis, and Western and Southern blot analysis. DNA was separated using agarose gel electrophoresis and visualized after staining in ethidium bromide. When required, the separated DNA was transferred onto nylon filters according to the method of Southern (1975). The nylon filter was probed with digoxigenin-labelled 1040 bp *aerA* PCR products or 597 bp *hlyA* PCR product for 18 h at 42 °C. Removal of residual unbound probes and detection of the DNA fragment using the digoxigenin detection system are described elsewhere (Wong *et al.*, 1997).

RESULTS

Cloning and sequencing of *hlyA* and *aerA*

A single cosmid, designated pCWH1, which was β-haemolytic on HBA was identified in a bank of 250 cosmids following cloning of *A. hydrophila* A6 *Sau3A*I-digested DNA into cosmid vector pHC79. Stepwise deletion on pCWH1 using enzymes *Cla*I and *Sph*I indicated that the gene for the expression of the haemolysin was located on a 2 kb *Cla*I–*Sph*I fragment. The DNA sequence of the insert of the resulting plasmid, pCWH3, was determined and the restriction map is shown in Fig. 1.

Fig. 1. Restriction endonuclease cleavage pattern, DNA labelling, and deletion analysis of plasmid pCWH3. The bold box on the right end of pCWH3 corresponds to residual cosmid vector pHC79 (Hohn & Collins, 1980). Primers H1 (5'-GGC CGG TGG CCC GAA GAT ACG GG-3') and H2 (5'-GGC GGC GCC GGA CGA GAC GGG-3') were used in a PCR reaction to detect a 597 bp *hlyA* DNA fragment in a PCR survey on *A. hydrophila* strains ATCC 7966 and Ah65. A digoxigenin-labelled *Sma*I probe was used in Southern blot analysis to confirm *A. hydrophila* chromosomal arrangements. pCWH4 and pCWH5 are the mutant constructs made by deletion of an 857 bp *Eag*I fragment of the *hlyA* gene.

For *aerA*, a 1040 bp fragment in the 3' region of the *A. hydrophila aerA* gene (Fig. 2) was amplified using primers A3 and A4. The purified PCR product was then cloned into pGEM-T (Promega) to yield pCWA1.

Sequence analysis

The nucleotide sequence of the *Cla*I–*Sph*I fragment of pCWH3 revealed an ORF (nt 172–2037) encoding a potential gene product comprising 621 amino acids with a molecular size of 69.0 kDa. The putative promoter regions of this ORF showed 83% identity (5/6 nucleotides) with consensus *Escherichia coli* –10 and –35 sequences. In addition, a putative ribosome-binding site (Shine & Dalgarno, 1974), cAAGGAGaT, was found seven bases upstream from the ATG start codon. Downstream from the termination codon at position 2037 is an area of dyad symmetry (nt 2063–2090) with a ΔG value of –18.90 kcal mol⁻¹ (–79.38 kJ mol⁻¹) which may act as a transcriptional attenuator. According to the criteria of von Heijne (1986), this ORF contains a putative signal peptide with putative cleavage sites between amino acids 20 and 21 and possibly 30 and 31.

Amino acid sequence identity and similarity

In this paper, the ORF encoding the 621-amino-acid protein is referred to as *hlyA*. The inferred amino acid sequence of HlyA showed 85% identity to the AH11 haemolysin of *A. hydrophila* ATCC 7966 (Hirono & Aoki, 1991), 75% identity to the ASH4 haemolysin of *A. salmonicida* 17-2 (Hirono & Aoki, 1993) and 51% identity to the HlyA haemolysin of *V. cholerae* El Tor strain O17 (Alm *et al.*, 1988). *hlyA* is therefore a distinct allele of this gene in *Aeromonas*.

Inactivation of the *hlyA* and *aerA* genes

The construction of the *A. hydrophila hlyA* deletion mutant is shown in Fig. 1. Briefly, the 857 bp *Eag*I DNA fragment of the *hlyA* gene in pCWH3 was deleted and the resulting 5943 bp fragment was recircularized to form pCWH4. The *Sma*I DNA fragment of pCWH4 containing the *Aeromonas* DNA flanking the deleted 857 bp fragment was then cloned into the *Sma*I-digested ends of the suicide plasmid pCACTUS2, yielding pCWH5. pCWH5 was then transferred by conjugation to *A. hydrophila* A6. The deletion mutant was designated A6H. Southern blot analysis of the A6H *Sma*I chromosomal digest confirmed the predicted chromosomal rearrangement in the *A. hydrophila* A6 genome (Fig. 3).

The process used to construct the *A. hydrophila aerA* insertionally inactivated mutant is illustrated in Fig. 2. An 850 bp non-polar *aphA-3* cassette with a kanamycin resistance marker from vector pUC18K (Menard *et al.*, 1993) was inserted into the *Sma*I site within the *aerA* gene of pCWA1 to yield pCWA2. The *Bam*HI fragment of pCWA2 containing the kanamycin cartridge

Fig. 2. Restriction endonuclease cleavage pattern, DNA labelling, and insertion analysis of the published aerolysin DNA sequence (Howard *et al.*, 1987). The arrows in the *aerA* box indicate the direction of transcription. Primers A1 (5'-GCC TGA GCG AGA AGG T-3') and A2 (5'-CAG TCC CAC CCA CTT C-3') were used in PCR to detect a 416 bp DNA fragment in a PCR survey on *A. hydrophila* strains ATCC 7966 and Ah65. A digoxigenin-labelled 1040 bp PCR product created by primers A3 (5'-CGC CTA TAA CCT GGA TCC CGA C-3') and A4 (5'-CTG ATC AGG TTC CAC GGA TCC-3') was used as a probe in Southern blot analysis to confirm *A. hydrophila* chromosomal arrangements. The triangle in pCWA2 and pCWA3 indicates the *Sma*I site where the non-polar kanamycin (Km^r) cartridge was inserted.

Fig. 3. Southern blot analysis of *hlyA* (A6H), *aerA* (A6A) and *hlyA aerA* (A6HA) mutants. The arrows indicate the confirmed chromosomal rearrangement. (a) *Sma*I-digested chromosomal DNAs were probed with digoxigenin-labelled *Sma*I fragment DNA of pCWA3. The 1.8 kb band in A6 *Sma*I was replaced by a 0.9 kb band in A6H and A6HA as a result of the 857 bp deletion during the making of the deletion construct. (b) *Bam*HI-digested chromosomal DNAs were probed with digoxigenin-labelled 1040 bp *aerA* PCR product. The 0.9 kb band in A6 *Bam*HI was replaced by a 1.8 kb band in A6A and A6HA as a result of the insertion of the kanamycin resistance cartridge.

Table 2. Virulence properties of *A. hydrophila* A6 mutants

Strain	Mean LD ₅₀ *		
A6	6.3×10^7 ($n = 5$)	++	1024
A6H	4.8×10^7 ($n = 2$) ($P > 0.05$)	++	1024
A6A	6.6×10^8 ($n = 2$) ($P > 0.05$)	+	8
A6HA	1.4×10^9 ($n = 3$) ($P < 0.05$)	0	< 4

* Numbers in parentheses indicate the number of times (n) the LD₅₀ was determined. The P values were determined from the Scheffe test.

† β -Haemolysis was observed as a transparent zone surrounding a bacterial colony on HBA following incubation at 30 °C for 18 h. ++, Strong β -haemolysis; +, weak β -haemolysis; 0, no β -haemolysis.

‡ Cytotoxicity was expressed as titres with < 4 indicating undetectable cytopathic effects.

with flanking *Aeromonas* DNA was then cloned into the *Bam*HI-digested pCACTUS2 to yield plasmid pCWA3. The mutant construct was then transferred by conjugation to *A. hydrophila* A6. The insertion mutant was designated A6A. Southern blot analysis of the A6A

*Bam*HI chromosomal digest confirmed the predicted chromosomal rearrangement in the *A. hydrophila* A6 genome (Fig. 3). Similarly, pCWA3 was also used in the construction of the *hlyA aerA* double mutant in *A. hydrophila* A6H to yield A6HA. Southern blot analysis

of the A6HA *Sma*I and *Bam*HI chromosomal digest confirmed the predicted chromosomal rearrangements in the *A. hydrophila* A6 genome (Fig. 3). In addition, both A6A and A6HA were resistant to kanamycin, suggesting that the 5' ribosome-binding site, start codon and translational reading frame of the cassette spliced in-frame with the 3' end of the disrupted gene.

Virulence of the *A. hydrophila* mutants in the suckling mouse model

To investigate whether the *A. hydrophila* mutants were attenuated, strains A6H, A6A and A6HA were assessed for virulence in suckling mice. All LD₅₀ determinations were performed at least twice and compared with the LD₅₀ of wild-type A6 (Table 2). Statistical analysis of the LD₅₀ results for all four strains showed that the results were significantly different (ANOVA, $P = 0.017$). This difference was only apparent between A6 and A6HA (Scheffe, $P > 0.05$), showing that the 20-fold increase in LD₅₀ was statistically significant. In the negative control group, mice administered 48 LD₅₀ of 65 °C heat-killed wild-type A6 did not show any ill-effects during the course of the experiment. In the positive control group, mice administered 48 LD₅₀ of *A. hydrophila* A6 died within 9 h post-infection.

To determine if the mice administered 48 LD₅₀ of A6HA died as a result of fluid accumulation in the gut, the FA ratio for 10 infected mice was determined at 9 h post-infection. As a positive control, the FA ratio for two surviving mice administered 48 LD₅₀ of wild-type A6 was 89 mg g⁻¹. Mice administered 48 LD₅₀ of A6HA yielded a mean FA ratio of 49 mg g⁻¹, a result similar to negative control mice administered broth only as demonstrated by Wong *et al.* (1996). Autopsy of mice infected with 48 LD₅₀ of wild-type A6 demonstrated extensive accumulation of fluid in the gut whereas mice administered 48 LD₅₀ of A6HA showed no pathological changes (data not shown).

Biological activity of the *A. hydrophila* mutants

To investigate whether the *A. hydrophila* haemolytic toxins are cytotoxins, the culture supernatants of A6, A6H, A6A and A6HA were analysed for cytotoxicity to the BGMK cell monolayer and the results were expressed as a cytotoxic titre (Table 2). The cytotoxic titre of both A6 and A6H was 1024. The cytotoxic titre of A6A decreased to 8, a 128-fold drop. The cytotoxic titre was eliminated at all dilutions in the A6HA double mutant. Both A6H and A6A were still haemolytic on blood agar. However, inactivation of both toxin genes in the A6HA mutant completely abrogated haemolytic activity on blood agar plates.

Distribution of the *hlyA* and *aerA* genes in *Aeromonas* isolates

Primers H1 and H2 (Fig. 1) were used to determine whether a 597 bp *hlyA* gene fragment was present in *A. hydrophila* strain Ah65. A similar survey was performed

by PCR using primers A1 and A2 (Fig. 2) to determine whether a 416 bp *aerA* gene fragment was detectable in *A. hydrophila* ATCC 7966. PCR results showed that the 597 bp *hlyA* and 416 bp *aerA* PCR products could be amplified from both strains (data not shown).

DISCUSSION

We report the mutagenesis of two distinct haemolysin genes for the first time in *A. hydrophila*. We suggest that the term HlyA be used to describe proteins which are homologous to the *V. cholerae* HlyA haemolysin and AerA for proteins homologous to aerolysin. Previously, terms such as haemolysin (Hirono & Aoki, 1991, 1993; Hirono *et al.*, 1992), aerolysin (Chakraborty *et al.*, 1987; Howard *et al.*, 1987) and cytolytic enterotoxin (Chopra *et al.*, 1993) were often used sometimes interchangeably for proteins of this type (Table 3). The amino acid homology demonstrated by ASA1 (66%) and AerA (77%) to the *A. hydrophila* Ah65 aerolysin and by ASH4 (75%) and AHH1 (85%) to the *A. hydrophila* A6 HlyA haemolysin, the gene of which is clearly a distinct allele of the genes of these previously described toxins, lends support to the suggestion of Husslein *et al.* (1991) that both haemolytic toxins are a heterogeneous family of cytolytic haemolysins (Table 3). This diversity resembles that observed for the family of Shiga- and Shiga-like toxins of *Shigella* and enteroinvasive *E. coli* (Jackson, 1990).

The *hlyA* and *aerA* gene products are significantly different from one another at the amino acid level (18% identity). A similar finding of two distinct haemolysin genes, ASH3 and ASH4, was also reported in a psychrophilic non-motile aeromonad, *A. salmonicida* 17-2 (Hirono & Aoki, 1993). The ASH3 haemolysin was 66% homologous to the aerolysin of *A. hydrophila* Ah65 (Hirono & Aoki, 1993), and is a homologue of AerA. The AHH1 and ASH4 haemolytic toxins had 50% and 45% (Hirono & Aoki, 1993) homology, respectively, to the HlyA haemolysin of *V. cholerae* O1 biotype El Tor O17.

The HlyA haemolysin was shown to be homologous to AHH1, ASH4 and *V. cholerae* HlyA haemolytic toxins. However, using the criteria of von Heijne (1986), the published ORFs for AHH1 and ASH4 haemolytic toxins did not have putative signal peptides. Analysis of the potential protein coding region upstream from the ATG start codons of AHH1 and ASH4 showed that the residues responsible for the putative signal peptides are potentially encoded but are not found within the putative ORF, possibly as a result of frameshift. It is possible that a sequencing error was responsible for this observation and the apparent lack of signal peptides in ASH4 and AHH1. The extracellular location of these proteins, and the presence of a possible signal peptide in the *A. hydrophila* A6 HlyA reported here and for the *V. cholerae* HlyA (Alm *et al.*, 1988; Yamamoto *et al.*, 1990) provide evidence that this is a reasonable explanation.

The mechanism for the secretion of the *A. hydrophila* HlyA haemolysin across the outer membrane after its

Table 3. Summary of published nomenclature and properties of β -haemolytic toxins of *Aeromonas* spp.

Gene/protein	Organism (source)	Molecular mass (kDa)	Identity with other proteins	Reference
Aerolysin	<i>A. hydrophila</i> Ah65 (rainbow trout isolate)	53.8	Original aerolysin	Howard <i>et al.</i> (1987)
AHH3	<i>A. hydrophila</i> 28SA (eel isolate)	54.7	94% identity with aerolysin	Hirono <i>et al.</i> (1992)
AHH5	<i>A. hydrophila</i> AH-1 (human isolate)	53.7	92% identity with aerolysin	Hirono <i>et al.</i> (1992)
Cytolytic enterotoxin	<i>A. hydrophila</i> SSU (human diarrhoeal isolate)	54.5	93% identity with aerolysin	Chopra <i>et al.</i> (1993)
ASA1	<i>A. sobria</i> 33 (human isolate)	53.9	66% identity with aerolysin	Hirono <i>et al.</i> (1992)
aerA	<i>A. trota</i> AB3 (human diarrhoeal isolate)	54.4	77% identity with aerolysin	Husslein <i>et al.</i> (1988)
ASH3	<i>A. salmonicida</i> 17-2 (fish isolate)	54.2	66% identity with aerolysin	Hirono & Aoki (1993)
AHH1	<i>A. hydrophila</i> ATCC 7966 (tinned milk isolate)	63.6	50% identity with <i>V. cholerae</i> HlyA	Hirono & Aoki (1991)
HlyA	<i>A. hydrophila</i> A6 (human diarrhoeal isolate)	69.0	51% identity with <i>V. cholerae</i> HlyA	This study
ASH4	<i>A. salmonicida</i> 17-2 (fish isolate)	63.4	45% identity with <i>V. cholerae</i> HlyA	Hirono & Aoki (1993)

translocation across the bacterial inner membrane is unknown. Recently, the HlyA haemolysin of *V. cholerae* has been shown to have a single channel conductance, suggesting that this haemolysin has a channel-forming property (Menzl *et al.*, 1996). It is probable by analogy that the *A. hydrophila* HlyA haemolysin is also a pore-forming haemolysin.

In contrast to the behaviour of an analogous protein in *V. cholerae*, the A6H mutant did not show attenuation in the suckling mouse model (Scheffe, $P > 0.05$) nor did it show significant reduction in cytotoxic or haemolytic activity (Table 2). It has been reported that expression of the HlyA haemolysin in *V. cholerae* is regulated by an 11.9 kDa protein designated HlyU (Williams *et al.*, 1993). Insertional inactivation of the *hlyU* gene inhibited production of the HlyA haemolysin and a 28 kDa Hcp (haemolysin coregulated protein) protein. This increased the LD₅₀ by 100-fold in a suckling mouse model (Williams *et al.*, 1996). The results would suggest that, in the suckling mouse model, the HlyA haemolysin is a more significant virulence factor in *V. cholerae* than the HlyA haemolysin is for *A. hydrophila*.

Inactivation of the *aerA* gene resulted in a ninefold increase in LD₅₀ in the suckling mouse model but the attenuation was not statistically significant (Scheffe, $P > 0.05$). Statistically significant attenuation was attained only when both HlyA and AerA production was negated, resulting in a 20-fold increase in LD₅₀ (Scheffe, $P < 0.05$). The attenuation of virulence conferred by A6HA was also accompanied by complete loss of haemolytic and cytotoxic activities (Table 2). In addition, at 9 h post-inoculation, no fluid accumulation was observed in the gut of mice administered 48 LD₅₀ of the non- β -haemolytic double mutant (mean FA ratio = 48 mg g⁻¹) whilst mice administered the same dose of

wild-type A6 had significant accumulation of fluid in the gut (mean FA ratio = 89 mg g⁻¹). These results are similar to those of Stelma *et al.* (1986), who showed that culture filtrates of 19/21 β -haemolytic *A. hydrophila* isolates elicited fluid accumulation in permanently ligated rabbit ileal loops, whereas no fluid accumulation was shown in filtrates of eight non- β -haemolytic isolates. In contrast, inactivation of the aerolysin gene alone in *Aeromonas trota* abrogated both haemolytic and cytotoxic activity (Chakraborty *et al.*, 1987). The *A. trota* mutant was attenuated (LD₅₀ > 10⁹) in an intraperitoneal mouse model in comparison with the wild-type strain, which had an LD₅₀ of 5×10^7 . The cytolytic enterotoxin of *A. hydrophila* SSU, which is 93% homologous to the aerolysin of *A. hydrophila* Ah65, has also been shown to be lethal to mice after the toxin was injected intravenously (Chopra *et al.*, 1993). These findings are consistent with the view that aeromonads are potential pathogens.

That reduction in virulence of the A6HA mutant, while significant, did not represent complete attenuation supports the notion that these gene products are major but not sole virulence factors. Other virulence-related factors such as pili (Atkinson & Trust, 1980), haemagglutinins (Atkinson *et al.*, 1987), lipopolysaccharide (Francki & Chang, 1994) and outer-membrane proteins (Quinn *et al.*, 1993) have been associated with virulence in aeromonads. The construction of multiple mutations for these factors and testing for further attenuation in the suckling mouse model would be a way of testing the relative contribution of these factors.

In *A. hydrophila* ATCC 7966 and *A. hydrophila* Ah65, both *hlyA* and *aerA* genes were detected by PCR. For *A. hydrophila* ATCC 7966, in addition to the published *hlyA* allele already reported, the *aerA* gene was also

detected. Similarly, for *A. hydrophila* Ah65, from which the aerolysin gene was cloned and sequenced, the *hlyA* gene was also detected by PCR. This supports the hypothesis that two toxins contribute to virulence in *A. hydrophila* and that the occurrence of the two toxin genes may be widespread in both clinical and environmental isolates within this species. A PCR survey for the detection of the *hlyA* and *aerA* toxin genes in other *A. hydrophila* isolates and *Aeromonas* isolates of other species is under way to determine their distribution and contribution to virulence in the suckling mouse model.

In conclusion, two haemolytic toxins are produced by *A. hydrophila*. Only when production of both HlyA and AerA was inactivated by mutagenesis was both cytotoxic and haemolytic activity eliminated and statistically significant but not total attenuation observed. Both haemolytic toxins contribute to virulence but factors other than haemolytic toxins contribute to virulence of *A. hydrophila*.

ACKNOWLEDGEMENTS

This project was supported by the University of South Australia, School of Pharmacy and Medical Sciences Research Fund. We thank the IMVS for allowing the use of reagents and equipment for performing the molecular biology experiments and Clive and Vera Ramaciotti Foundations for the purchase of the Corbett thermal sequencer. We also wish to thank Phillip Leppard for his statistical consultation (Department of Statistics, University of Adelaide).

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Received 21 May 1997; revised 30 September 1997; accepted 23 October 1997.