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

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**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; Delamorena *et al.*, 1993; Ogunsanya *et al.*, 1994; Rautelin *et al.*, 1993; 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
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 aero-lysin was originally cloned and sequenced from *A. hydrophila* ATCC 7966 (Hirono & Aoki, 1991). The gene 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 μg vitamin B, 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

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

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>A. hydrophila</em> strains</td>
<td>Wild-type; Ap'</td>
<td>Hirono &amp; Aoki (1991)</td>
</tr>
<tr>
<td>ATCC 7966</td>
<td>Wild-type; Ap'</td>
<td>Howard et al. (1987)</td>
</tr>
<tr>
<td>Ah65</td>
<td>Wild-type; Ap'</td>
<td>Atkinson &amp; Trust (1980)</td>
</tr>
<tr>
<td>A6</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>A6H</td>
<td>blyA Eagl deletion mutation</td>
<td>This study</td>
</tr>
<tr>
<td>A6A</td>
<td>aerA Km' insertion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>A6HA</td>
<td>blyA aerA mutant</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Ap' cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pUC18K</td>
<td>pUC18 with the non-polar apha-3 Km' resistance cassette</td>
<td>Menard et al. (1993)</td>
</tr>
<tr>
<td>pCACTUS2</td>
<td>Suicide Km' cloning vector, contains sacB and has a temperature-sensitive origin of replication</td>
<td>C. Clark and others (unpublished)</td>
</tr>
<tr>
<td>pCWH1</td>
<td>pHC79 carrying an approximately 40 kb fragment of <em>A. hydrophila</em> A6 genomic DNA</td>
<td>This study</td>
</tr>
<tr>
<td>pCWH2</td>
<td>Sphl stepwise deletion of pCWH1</td>
<td>This study</td>
</tr>
<tr>
<td>pCWH3</td>
<td>ClaI stepwise deletion of pCWH2</td>
<td>This study</td>
</tr>
<tr>
<td>pCWH4</td>
<td>pCWH3 with an 857 bp Eagl fragment of blyA deleted</td>
<td>This study</td>
</tr>
<tr>
<td>pCWH5</td>
<td>pCACTUS2 carrying the SmaI fragment of pCWH4 containing the deletion mutation</td>
<td>This study</td>
</tr>
<tr>
<td>pCWA1</td>
<td>pGEM-T carrying the 1040 bp PCR product derived from aerA</td>
<td>This study</td>
</tr>
<tr>
<td>pCWA2</td>
<td>pCWA1 carrying a Km' gene in the SmaI site</td>
<td>This study</td>
</tr>
<tr>
<td>pCWA3</td>
<td>pCACTUS2 carrying the BamHI fragment of pCWA2</td>
<td>This study</td>
</tr>
</tbody>
</table>
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 BGMK 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 \( A. \) \( \text{hydrophila} \) A6 Sau3AI-digested DNA into cosmid vector pH79. Stepwise deletion on pCWH1 using enzymes ClaI and SpH1 indicated that the gene for the expression of the haemolysin was located on a 2 kb ClaI–SpH1 fragment. The DNA sequence of the insert of the resulting plasmid, pCWH3, was determined and the restriction map is shown in Fig. 1.

For aerA, a 1040 bp fragment in the 3' region of the \( A. \) \( \text{hydrophila} \) 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 ClaI–SpH1 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 \( Escherichia \) \( \text{coli} \) −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 AHH1 haemolysin of \( A. \) \( \text{hydrophila} \) ATCC 7966 (Hirono & Aoki, 1991), 75% identity to the ASH4 haemolysin of \( A. \) \( \text{salmonicida} \) 17-2 (Hirono & Aoki, 1993) and 51% identity to the HlyA haemolysin of \( V. \) \( \text{cholerae} \) El Tor strain O17 (Alm et al., 1988). hlyA is therefore a distinct allele of this gene in \( A. \) \( \text{hydrophila} \).

**Inactivation of the hlyA and aerA genes**

The construction of the \( A. \) \( \text{hydrophila} \) hlyA deletion mutant is shown in Fig. 1. Briefly, the 857 bp EagI DNA fragment of the hlyA gene in pCWH3 was deleted and the resulting 5943 bp fragment was recircularized to form pCWH4. The SmaI DNA fragment of pCWH4 containing the \( A. \) \( \text{hydrophila} \) DNA flanking the deleted 857 bp fragment was then cloned into the SmaI-digested ends of the suicide plasmid pACTUS2, yielding pCWH5. pCWH5 was then transferred by conjugation to \( A. \) \( \text{hydrophila} \) A6. The deletion mutant was designated A6H. Southern blot analysis of the A6H SmaI chromosomal digest confirmed the predicted chromosomal rearrangement in the \( A. \) \( \text{hydrophila} \) A6 genome (Fig. 3).

The process used to construct the \( A. \) \( \text{hydrophila} \) 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 SmaI site within the aerA gene of pCWA1 to yield pCWA2. The BamHI 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 Smal site where the non-polar kanamycin (Km') cartridge was inserted.

Table 2. Virulence properties of A. hydrophila A6 mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean LD&lt;sub&gt;50&lt;/sub&gt;*</th>
<th>P&lt;sub&gt;H&lt;/sub&gt;</th>
<th>P&lt;sub&gt;B&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6</td>
<td>6.3 x 10&lt;sup&gt;7&lt;/sup&gt; (n = 5)</td>
<td>++</td>
<td>1024</td>
</tr>
<tr>
<td>A6H</td>
<td>4.8 x 10&lt;sup&gt;7&lt;/sup&gt; (n = 2) (P &gt; 0.05)</td>
<td>++</td>
<td>1024</td>
</tr>
<tr>
<td>A6A</td>
<td>6.6 x 10&lt;sup&gt;8&lt;/sup&gt; (n = 2) (P &lt; 0.05)</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>A6HA</td>
<td>1.4 x 10&lt;sup&gt;9&lt;/sup&gt; (n = 3) (P &lt; 0.05)</td>
<td>0</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate the number of times (n) the LD<sub>50</sub> was determined. The P values were determined from the Scheffe test.

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

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

with flanking Aeromonas DNA was then cloned into the BamHI-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 BamHI 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 Smal and BamHI 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 sucking mouse model**

To investigate whether the A. hydrophila mutants were attenuated, strains A6H, A6A and A6HA were assessed for virulence in sucking mice. All LD$_{50}$ determinations were performed at least twice and compared with the LD$_{50}$ of wild-type A6 (Table 2). Statistical analysis of the LD$_{50}$ 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 LD$_{50}$ was statistically significant. In the negative control group, mice administered 48 LD$_{50}$ 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 LD$_{50}$ of A. hydrophila A6 died within 9 h post-infection.

To determine if the mice administered 48 LD$_{50}$ 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 LD$_{50}$ of wild-type A6 was 89 mg g$^{-1}$. Mice administered 48 LD$_{50}$ 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 LD$_{50}$ of wild-type A6 demonstrated extensive accumulation of fluid in the gut whereas mice administered 48 LD$_{50}$ 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 ASAl (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
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 A6H1 mutant did not show attenuation in the suckling mouse model (Scheffé, *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 (Scheffé, *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 (Scheffé, *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 > 10⁶) in an intraperitoneal mouse model in comparison with the wild-type strain, which had an LD₅₀ of 5 × 10³. 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 pil (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

<table>
<thead>
<tr>
<th>Gene/protein</th>
<th>Organism (source)</th>
<th>Molecular mass (kDa)</th>
<th>Identity with other proteins</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerolysin</td>
<td><em>A. hydrophila</em> Ah65 (rainbow trout isolate)</td>
<td>53·8</td>
<td>Original aerolysin</td>
<td>Howard et al. (1987)</td>
</tr>
<tr>
<td>AH3</td>
<td><em>A. hydrophila</em> 28SA (eel isolate)</td>
<td>54·7</td>
<td>94% identity with aerolysin</td>
<td>Hirono et al. (1992)</td>
</tr>
<tr>
<td>AH5</td>
<td><em>A. hydrophila</em> AH-1 (human isolate)</td>
<td>53·7</td>
<td>92% identity with aerolysin</td>
<td>Hirono et al. (1992)</td>
</tr>
<tr>
<td>Cytolytic enterotoxin</td>
<td><em>A. hydrophila</em> SSU (human diarrhoeal isolate)</td>
<td>54·5</td>
<td>93% identity with aerolysin</td>
<td>Chopra et al. (1993)</td>
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<td>ASA1</td>
<td><em>A. sobria</em> 33 (human isolate)</td>
<td>53·9</td>
<td>66% identity with aerolysin</td>
<td>Hirono et al. (1992)</td>
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<tr>
<td>aerA</td>
<td><em>A. trota</em> AB3 (human diarrhoeal isolate)</td>
<td>54·4</td>
<td>77% identity with aerolysin</td>
<td>Husslein et al. (1988)</td>
</tr>
<tr>
<td>ASH3</td>
<td><em>A. salmonicida</em> 17-2 (fish isolate)</td>
<td>54·2</td>
<td>66% identity with aerolysin</td>
<td>Hirono et al. (1992)</td>
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<tr>
<td>AHH1</td>
<td><em>A. hydrophila</em> ATCC 7966 (tinned milk isolate)</td>
<td>63·6</td>
<td>50% identity with <em>V. cholerae</em> HlyA</td>
<td>Hirono &amp; Aoki (1993)</td>
</tr>
<tr>
<td>HlyA</td>
<td><em>A. hydrophila</em> A6 (human diarrhoeal isolate)</td>
<td>69·0</td>
<td>51% identity with <em>V. cholerae</em> HlyA</td>
<td>This study</td>
</tr>
<tr>
<td>ASH4</td>
<td><em>A. salmonicida</em> 17-2 (fish isolate)</td>
<td>63·4</td>
<td>45% identity with <em>V. cholerae</em> HlyA</td>
<td>Hirono &amp; Aoki (1993)</td>
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</tbody>
</table>
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*.

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