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

Christopher Y. F. Wong,† Michael W. Heuzenroeder and Robert L. P. Flower

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 AHHl 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; 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

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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

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
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<tr>
<td><strong>A. hydrophila</strong></td>
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<td></td>
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<tr>
<td>strains</td>
<td></td>
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</tr>
<tr>
<td>ATCC 7966</td>
<td>Wild-type; Ap'</td>
<td>Hirono &amp; Aoki (1991)</td>
</tr>
<tr>
<td>Ah65</td>
<td>Wild-type; Ap'</td>
<td>Howard et al. (1987)</td>
</tr>
<tr>
<td>A6</td>
<td>Wild-type; Ap'</td>
<td>Atkinson &amp; Trust (1980)</td>
</tr>
<tr>
<td>A6H</td>
<td>hlyA EagI 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>hlyA aerA mutant</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<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 Cm' 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 A. hydrophila A6 genomic DNA</td>
<td>This study</td>
</tr>
<tr>
<td>pCWH2</td>
<td>SphI stepwise deletion of pCWH1</td>
<td>This study</td>
</tr>
<tr>
<td>pCWH3</td>
<td>Clal stepwise deletion of pCWH2</td>
<td>This study</td>
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<td>pCWH4</td>
<td>pCWH3 with an 857 bp EagI fragment of hlyA deleted</td>
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</tr>
<tr>
<td>pCWH5</td>
<td>pCACTUS2 carrying the Smal fragment of pCWH4 containing the deletion mutation</td>
<td>This study</td>
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<tr>
<td>pCWA1</td>
<td>pGEM-T carrying the 1040 bp PCR product derived from aerA</td>
<td>This study</td>
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<tr>
<td>pCWA2</td>
<td>pCWA1 carrying a Km' gene in the Smal 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>

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 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 LD<sub>50</sub> 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 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. hydrophila A6 Sau3AI-digested DNA into cosmid vector pHC79. Stepwise deletion on pCWH1 using enzymes ClaI and SphI indicated that the gene for the expression of the haemolysin was located on a 2 kb ClaI–SphI 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.](image)

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 ClaI–SphI fragment of pCWH3 revealed an ORF (nt 172–2037) encoding a potential gene product comprising 621 amino acids with a molecular size of 690 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 –1890 kcal mol⁻¹ (–7938 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. 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 EagI DNA fragment of the hlyA gene in pCWH3 was deleted and the resulting 5943 bp fragment was recircularized to form pCW4. The SmaI DNA fragment of pCW4 containing the Aeromonas DNA flanking the deleted 857 bp fragment was then cloned into the SmaI-digested ends of the suicide plasmid pCACTUS2 yielding pCWH5. pCW4 was then transferred by conjugation to A. 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. 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 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 CTG T-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.

Fig. 3. Southern blot analysis of hlyA (A6H), aerA (A6A) and hlyA aerA (A6HA) mutants. The arrows indicate the confirmed chromosomal rearrangement. (a) Smal-digested chromosomal DNAs were probed with digoxigenin-labelled Smal fragment DNA of pCWH3. The 1.8 kb band in A6 Smal 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) BamHI-digested chromosomal DNAs were probed with digoxigenin-labelled 1040 bp aerA PCR product. The 0.9 kb band in A6 BamHI 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean LD&lt;sub&gt;50&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>
</tr>
<tr>
<td>A6H</td>
<td>4.8 x 10&lt;sup&gt;7&lt;/sup&gt; (n = 2) (P &gt; 0.05)</td>
</tr>
<tr>
<td>A6A</td>
<td>6.6 x 10&lt;sup&gt;8&lt;/sup&gt; (n = 2) (P &gt; 0.05)</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>
</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.

† 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 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 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 ATCC7966. 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 AH1 (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 Hussels 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 AH1 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 AH1, ASH4 and V. cholerae HlyA haemolytic toxins. However, using the criteria of von Heijne (1986), the published ORFs for AH1 and ASH4 haemolytic toxins did not have putative signal peptides. Analysis of the potential protein coding region upstream from the ATG start codons of AH1 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 AH1. 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 A6HA 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 LD<sub>50</sub> by 100-fold in a sucking mouse model (Williams et al., 1996). The results would suggest that, in the sucking 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 LD<sub>50</sub> in the sucking 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 LD<sub>50</sub> (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 LD<sub>50</sub> of the non-β-haemolytic double mutant (mean FA ratio = 48 mg g<sup>-1</sup>) whilst mice administered the same dose of wild-type A6 had significant accumulation of fluid in the gut (mean FA ratio = 89 mg g<sup>-1</sup>). 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 (LD<sub>50</sub> > 10<sup>8</sup>) in an intraperitoneal mouse model in comparison with the wild-type strain, which had an LD<sub>50</sub> of 5 × 10<sup>7</sup>. 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 (Franck & 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 sucking 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

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

<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>AHH3</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>AHH5</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. hydrophilia</em> SSU (human diarrhoeal isolate)</td>
<td>54.5</td>
<td>93% identity with aerolysin</td>
<td>Chopra et al. (1993)</td>
</tr>
<tr>
<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>
</tr>
<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 &amp; Aoki (1993)</td>
</tr>
<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 (1991)</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>
</tr>
</tbody>
</table>
detected. Similarly, for \textit{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 \textit{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 \textit{A. hydrophila} isolates and \textit{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 \textit{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 \textit{A. hydrophila}.

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**REFERENCES**


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