The role of aerolysin in *Aeromonas*-associated infections

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The cytotoxic haemolysin, aerolysin, has been implicated as an important virulence factor in *Aeromonas*-associated infections[1,5,6]. Wild type isolates vary widely in their ability to produce the toxin, and toxicity assays performed with mice show a correlation between amount of toxin produced and mortality[4]. More recently, a study of 686 isolates from various geographical locations has shown a strong correlation between hemolysis and enterotoxicity[2].

In order to study the role of the toxin in *Aeromonas*-associated infections, we have cloned the corresponding gene from a clinical isolate. The aerolysin gene (*aer* A) was identified from a gene bank carrying DNA from strain AH 2, by screening for hemolytic activity on blood agar plates and cytotoxicity to Chinese Hamster ovary cells. Genetic mapping of the cloned fragment by transposon mutagenesis with Tn *1000* and deletion analysis with restriction endonucleases allowed us to define flanking regions which modulate the synthesis (*aer* B) and expression (*aer* C) of the aerolysin gene (*aer* A)[3].

The pathogenic potential of aerolysin was evaluated by constructing isogenic mutants of AH 2 which carry specific deletions within the *aer* determinant on the chromosome. Our strategy for obtaining such mutants was to introduce deleted derivatives of the *aer* determinant, cloned onto pMB1-based mobilizable vectors, into *A. hydrophila* AH 2. These vectors are unable to replicate stably in AH 2 and are rapidly lost by segregation. In order to detect mutants, association of a selectable marker with the cloned DNA sequence was required. The kanamycin resistance gene from the transposon Tn *903* was used for this purpose.

The plasmid pHPC3-702 carries a 1.4 kilobase(kb) Kpn I fragment encoding resistance to kanamycin in place of the 1.8 kb fragment from the *aer* determinant on plasmid pHPC3-700. *E. coli* recombinants harboring pHPC3-702 are completely devoid of both hemolytic and cytotoxic activities. A marker exchange procedure was used to recombine the deletion-*kan*^{r}-substitution on pHPC3-702 into the chromosome of a spontaneously derived nalidixic acid-resistant strain of AH 2 (AB3). Subsequent in vivo recombination and segregation of the plasmid produced genetic recombinants that had the resident *aer* A *aer* B region replaced by the substitution mutation carried on pHPC3-702 (fig. 1, A and B). One such recombinant, AB3 *aer*-5 was used in subseqeunt studies. DNA hybridization was used to confirm the genetic structure of AB3 *aer*-5. Immunoblots performed with specific antisera directed against aerolysin showed total absence of aerolysin in both cell lysates and supernatant fluids of AB3 *aer*-5.

We next determined the 50% lethal dose (LD_{50}) of both the parental strain and its isogenic aerolysin-negative derivative. The results are depicted in the table. Intraperitoneal injection of mice with the parental strain AB3 showed a LD_{50} of around 5×10^7 cells, while the aerolysin-negative strain had a LD_{50} of more than 8×10^8 cells. To exclude the possibility that unlinked mutations affecting toxicity had been introduced during the construction of AB3 *aer*-5, we reintroduced the wild type *aer* determinant into this strain. A double cross-over event leading to integration of the wild type *aer* A *aer* B genes was detected by plating on blood agar plates and scoring for hemolytic colonies. Several stable transconjugants were obtained, and one such

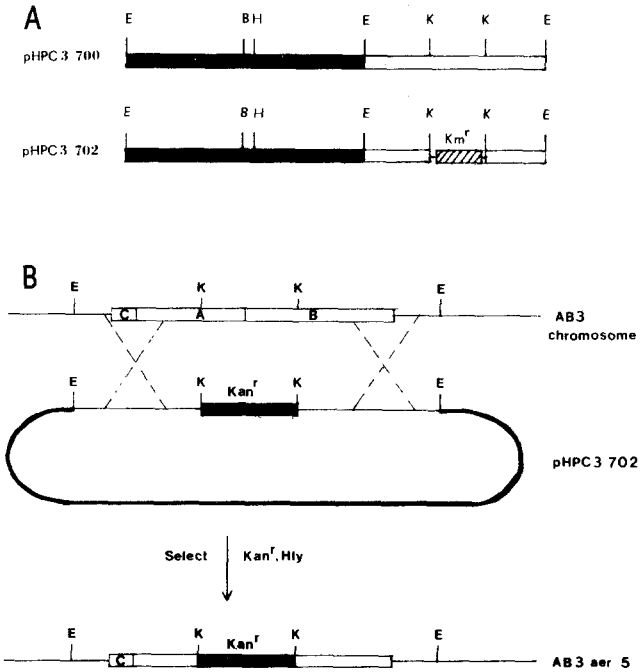

Figure 1. *A* Physical map of plasmid pHPC3-700 and pHPC3-702. The 1.8 kb *Kpn*I fragment of *Aeromonas* DNA has been replaced by a 1.4 kb *Kpn*I fragment encoding resistance to kanamycin in plasmid pHPC3-702. ■, vector pBR325 DNA sequences; □, *A. hydrophila* DNA sequences; ▨, Tn*903* DNA sequences; B = *Bam*HI; E = *Eco*RI; H = *Hind*III; K = *Kpn*I. *B* Construciton of the aerolysin-negative strain AB3 *aer*-5. A double cross-over event occuring between flanking homologous regions lead to the replacement of the normal *aer* A *aer* B region by the kan^r deletion-substitution.

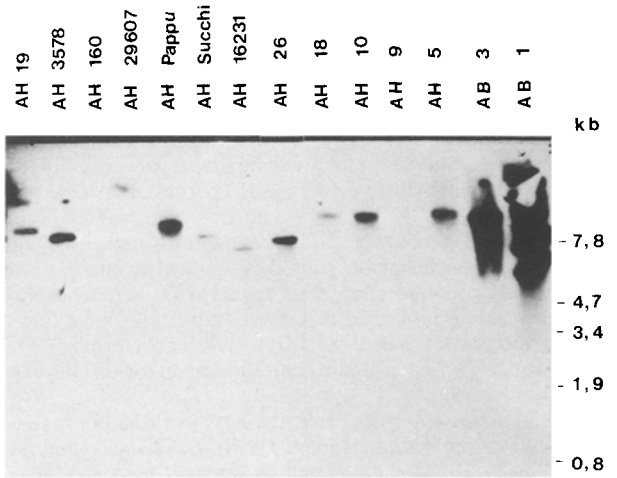

Figure 2. Southern blot analysis of the aerolysin gene in chromosomal DNA of various hemolytic *A. hydrophila* strains. Chromosomal DNA of all strains were digested with *Hind*III which does not cut within *aer* A. The multiple bands seen with chromosomal DNA of AB1 is a result of partially digested DNA; hybridization to AH9 DNA is barely visible in the figure.

transconjugant (AB3 *aer*-5 *rev*-1) was checked for its genetic structure using DNA hybridization and for aerolysin production in hemolysin and cytotoxin assays. As seen in the table, full toxicity was regained with the strain AB3 *aer*-5 *rev*-1 which had regained a LD_{50} of 5×10^7 cells.

All deaths of mice were recorded 30 h post inoculation; the majority of mice succumbed 18 h after i.p. infection. Pure cultures of *A. hydrophila* AB3 could be isolated from individually homogenized livers (5×10^7 cells/gm) and spleens (3×10^7 cells/gm) of dead mice. High concentrations of bacteria were also detected in blood samples of these mice. No loss of antibiotic markers encoded by AB3 (Nal^r) or AB3 *aer*-5 (Nal^r, Kan^r) could be detected upon plating onto selective/non-selective plates. These results clearly demonstrate the association of the toxin with lethality in mice and extends previous results obtained with wild type isolates producing varying amounts of aerolysin[7]. The systemic infection obtained with the mouse model is rapid, reflecting well the rapid course of wound infections and septicemia seen with human infections.

In order to test the ubiquity of the toxin in hemolytic *Aeromonas* strains we have used DNA hybridization analysis to probe for the presence of the gene. A 1.2 kb *Sma* I fragment, internal to the aerolysin gene, was labeled by nick-translation and used for hybridization to chromosomal DNA isolated from various hemolytic strains. All strains were hemolytic on both sheep and human blood agar plates, with the exception of strains AH 160 and AH 29607 which were hemolytic only on sheep blood agar plates. The results are shown in figure 2. All strains with the exception of AH 160 and AH 29607 hybridize to the aerolysin DNA probe (the hybridization to strain AH 9 is poorly seen). Clearly, there are hemolytic factor(s), other than aerolysin, present in *Aeromonas* species; it is also likely that a single strain may harbor more than one hemolytic activity. The other conclusion that can be drawn from the data is that the gene is associated with a different *Hind* III restriction fragment in independent isolates. This pattern has also been observed when the chromosomal DNA is restricted with the enzymes *Sma* I, *Bam* HI, *Eco* RI or *Kpn* I (data not shown). These results suggest a variable location of the aerolysin gene on the chromosome of independent isolates of *A. hydrophila*.

In summary, we have used the cloned aerolysin gene to introduce specific deletions into the chromosome of a clinical isolate of *A. hydrophila* AH 2. Mouse lethality studies with the parental strain and its isogenic mutant point to an important role of the toxin in systemic infections. DNA hybridization analysis indicates that the gene is unique and is present in variable locations on the chromosome of independent isolates. The presence of hemolytic factors other than aerolysin in *Aeromonas* species is suggested by the fact that not all hemolytic strains hybridize with the aerolysin gene probe.

Effect of the strains AB3, AB3 *aer*-5, and AB3 *aer*-5 *rev*-1 on mouse lethality

Strain	No. of cells injected	No. of mice dead/ No. tested
AB3	8×10^8	5/5
	6×10^8	9/9
	4×10^8	5/5
	2×10^8	5/5
	1×10^8	3/5
	5×10^7	1/5
AB3 *aer*-5	8×10^8	0/5
	6×10^8	1/9
	4×10^8	0/5
	2×10^8	0/5
	1×10^8	0/5
	5×10^7	0/5
AB3 *aer*-5 *rev*-1	8×10^8	5/5
	6×10^8	5/5
	4×10^8	5/5
	2×10^8	5/5
	1×10^8	3/5
	5×10^7	2/5

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Surface structure of pathogenic *Aeromonas*

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Key words. Aeromonas; pathogens; lipopolysaccharide; S-layers; proteins; surfaces; fish disease.

Macromolecular arrays of protein subunits known as surface protein arrays or S-layers are found on the outermost surfaces of a wide range of bacteria. In the case of pathogenic bacteria they interface with the host, and so are ideally placed to play important roles in pathogenesis. Virulent strains of *Aeromonas salmonicida* produce a protein array known as A-layer. The A-layer is composed of tetragonally arranged subunits of molecular weight (MW) 50,000[5]. Computer image processing of electron micrographs of negatively stained A-layer has shown that it contains two square patterns having p4 symmetry. Both square arrays are composed of two different morphological units arranged alternatively to give a face-centered lattice in which the four nearest neighbours of each unit are the other type of unit[7]. The lattice constant is slightly but significantly different in the two patterns,