BACTERIAL PATHOGENICITY

Characterisation of a type IV pilus produced by *Aeromonas caviae*

S. M. KIROV, K. SANDERSON and T. C. DICKSON

Division of Pathology, University of Tasmania, Hobart, Tasmania 7001, Australia

A pilus produced by a clinical isolate of *Aeromonas caviae* (strain CA195) was purified and partially characterised. The M_r of the pilin was estimated to be 23 kDa by SDS-PAGE. Its N-terminal amino-acid sequence showed that it was closely related to ‘bundle-forming’ type IV pili purified from other *Aeromonas* spp. associated with gastro-enteritis and considered to be important intestinal colonisation factors. Bundle-forming pili, often with a polar location, were seen on the surface of strain CA195 which was highly adherent to HEp-2 cells. Removal of surface structures by mechanical means decreased adhesion (by $\geq 50\%$) suggesting that these pili played some role in HEp-2 cell binding. This pilus type could prove an important marker for enteropathogenic *A. caviae* which appear to lack other putative virulence factors.

Introduction

Aeromonas caviae is one of the three *Aeromonas* spp. most frequently associated with gastro-enteritis, particularly in paediatric patients and travellers [1–3]. Despite this, its enteropathogenicity has been controversial. Strains of *A. caviae* reportedly lack virulence factors found in diarrhoea-associated *A. hydrophila* and *A. veronii* biovar *sobria* [4, 5], although it has been postulated that exotoxins may be produced under different assay conditions to those found optimal for these other *Aeromonas* spp. [6, 7].

Adhesion to the intestinal mucosa is likely to be a major determinant of pathogenicity. There are several reports that diarrhoea-associated isolates of *A. caviae* are highly adherent to human cell lines used as intestinal cell models [8–10]. Filamentous structures have been implicated, but adherence-related extracellular appendages have not yet been isolated or characterised from *A. caviae* [10, 11]. This study examined diarrhoea-associated isolates of *A. caviae* from Australian patients for putative virulence-associated properties that might help delineate enteropathogenicity.

Materials and methods

Bacterial strains

Twenty-two clinical isolates of *A. caviae*, isolated in Queensland in 1993 (Mr M. Tandy, Sullivan, Nico-

laides and Partners, Private Pathology, Taringa, Queensland, Australia) were included in this study. Twenty isolates were from faeces of patients with severe or persistent diarrhoea. Eight of the patients with diarrhoeal symptoms were infants ≤ 2 years old, two were ≥ 55 years old, four were aged between 2 and 55 years, and details of patient age were not available for the remaining six patients. All isolates had the *A. caviae* phenotype [12]. The strain from which pili were purified (strain CA195) was isolated from a 1-year-old male infant with severe diarrhoea. Its speciation (hybridisation group 4) was also confirmed by genetic typing (rRNA gene restriction patterns) by Dr M. Altwegg, University of Zürich, Switzerland [13]. Short-term storage of isolates was in minimal maintenance medium at room temperature [14]; long-term storage was in glycerol-peptone (1:4 glycerol: bacteriological peptone 1%, Oxoid L37) at -80°C .

Exotoxin assays

Full details of these assays are given elsewhere [14, 15]. Briefly, bacteria were cultured (37°C , 24 h with shaking) in tryptone soya broth supplemented with yeast extract L21 (Oxoid 6 g/L) (TSBY). Bacteria-free broth supernates from these cultures were assessed for haemolytic activity against rabbit red blood cells, enterotoxic activity in suckling mice and cytotoxic activity for Vero cells. Supernates from bacteria grown under different culture conditions, including double strength trypticase soya broth with no glucose ($2 \times \text{TSBGlu-}$) [16] and iron limitation (TSBY containing deferoxamine mesylate 100 $\mu\text{g/ml}$) [17], were also tested in these assays.

Adhesion assay

Isolates (1×10^6 cfu) were examined for their ability to adhere to HEp-2 cells after growth in TSBY at 22°C for 48 h [18]. Each isolate was assessed on triplicate coverslip cultures of semi-confluent HEp-2 cells on two or more occasions. Strains with a mean number of 5–10 bacteria/cell were defined as ‘adherent’, those with ≥ 10 bacteria/cell as ‘highly adherent’.

Electron microscopy

Bacterial cells and purified pili preparations were absorbed on to formvar-coated copper grids and negatively stained (uranyl acetate 1%, 30 s). They were examined with a Philips 410 electron microscope [19].

Purification of pili

Bacteria were grown in static TSBY cultures at room temperature ($\sim 20^\circ\text{C}$) for 2 days. They were harvested from 5×1 -L cultures, resuspended in 100 ml of 0.05 M Tris-HCl (pH 7.4) and chilled on ice. They were stirred with a magnetic stirrer (Magnetstir, Lab-Line Instruments, Melrose Park, IL, speed 4.5) for 2 h (4°C) to remove loosely associated material. Bacteria recovered by centrifugation (2000 g, 30 min) were resuspended in 60 ml of 0.05 M Tris-HCl. Pili were removed from the bacterial cell surface with an Omni-mixer (Omni International, Waterbury, CT, USA) at speed 3–4 for 10 s at 10-s intervals with intermittent cooling for 2 min. After centrifugation (26 000 g, 30 min), the supernate was filtered through a $0.2\text{-}\mu\text{m}$ pore membrane (Gelman Sciences). Proteins in the final supernate were precipitated with polyethylene glycol 6000 (PEG; Rhône-Polenc, Manchester) 1% and NaCl 5.8% and held at 4°C overnight. Precipitated proteins were collected by centrifugation (26 000 g, 30 min) and resuspended in 0.05 M Tris buffer. The precipitation procedure and foregoing cycle was repeated on the resulting supernate with PEG 2% and then 6%. The 1, 2 and 6% protein precipitates were examined by electron microscopy and SDS-PAGE. Pili in the PEG 2% precipitate were further purified by suspending the precipitate in 10 ml of 0.05 M Tris-HCl and repeating the cycle of PEG precipitation described above.

SDS-PAGE analysis of crude pili preparations

SDS-PAGE was performed as described by Laemmli [20] in a discontinuous acrylamide 15% gel (Mini-PROTEAN II, BioRad, USA).

N-terminal amino-acid sequence analysis

Proteins were transferred from SDS-PAGE gels to polyvinylidene difluoride (PVDF) membrane (Mini-Transblot cell, BioRad) and visualised with amido black (1% w/v in methanol 40% v/v, acetic acid 1% v/v). They were sequenced by automated Edman degradation employing a Porton P12090 equipped with

‘on-line’ PTH-amino-acid analysis (Macquarie University Centre for Analytical Biotechnology, Sydney, Australia).

Results

Toxin production and adhesion by *A. caviae* isolates

While broth supernates from a control strain of *A. veronii* biovar *sobria* grown under standard conditions had haemolytic titres ≥ 1024 , were strongly cytotoxic for Vero cells and elicited positive suckling mouse responses, supernates from all 22 *A. caviae* isolates were negative for haemolysin and enterotoxin and four only were weakly cytotoxic for Vero cells. Toxic activities did not increase when *A. caviae* strains were grown in $2 \times$ TSBGlu-, or under iron-limitation, for 20–24 h. However, 55% (12 of 22) of the isolates were adherent to HEp-2 cells, seven isolates being highly adherent. Adherent strains showed diffuse binding (uniform distribution over the cell surface) (Fig. 1).

Surface structures on adherent clinical strains of *A. caviae*

Electron microscopy of adherent strains showed that the *A. caviae* isolates were generally poorly piliated (growth in TSBY, 22°C , 48 h), with only a few (usually < 10) long, wavy pili present on each bacterial cell. Growth of selected environmental and clinical strains at low temperature (4°C) did not increase pili expression as was found for many strains of *A. veronii* biovar *sobria* [19]. Bundle-forming pili were seen after culture at 22°C on a small proportion (5–10%) of the bacterial population of one highly adherent clinical isolate (strain CA195) (Fig. 2A, B and C). These bundle-forming pili (Bfp) often had a polar location and were sometimes seen closely associated with the flagellum (Fig. 2B and C). Removal of pili from this strain by mechanical blending (Omni-mixer, speed 3–4 for

Fig. 1. Adhesion of *A. caviae* to HEp-2 cells. *A. caviae* strains grown in tryptone soya broth containing yeast extract 0.6% at 22°C for 48 h had a diffuse adherence pattern characterised by a uniform distribution of bacteria over the cell surface. Bar, $12\text{ }\mu\text{m}$.

Fig. 2. Transmission electron micrographs showing negatively stained, filamentous surface structures of *A. caviae* strain CA195 after culture in tryptone soya broth containing yeast extract 0.6% at 22°C for 48 h. (A) Shows flagellum (F), an isolated long, wavy pilus (LW) and bundles of long, thin, flexible pili (Bfp). Bfp were seen on a small proportion (5–10%) of bacteria of some adherent strains. Bar, 1.6 µm. (B) and (C) The long, flexible, bundle-forming pili of strain CA195 tended to have a polar location and were often seen closely associated with the single polar flagellum (F). Bars, 3.3 µm and 2.6 µm.

5 min) decreased adhesion to HEP-2 cells by > 50%. Therefore, experiments were done to purify and characterise the pili of this *A. caviae* isolate.

Purification and characterisation of filamentous surface structures of strain CA195

Pili from *A. caviae* strain CA195 proved more difficult to purify than the pili of an *A. veronii* biovar *sobria* strain (strain BC88) previously studied [21]. Unlike the latter strain, with which a discrete pilin subunit band was readily identified on SDS-PAGE analysis, preparations from the *A. caviae* strain resulted in multiple (> 15) protein bands, with several bands in the M_r region of type IV pilins (17–25 kDa). Modifications of the blending procedure (shorter blending time, reduced speed) did not markedly affect this protein band pattern. Electron microscopy of the PEG 1%, 2% and 6% precipitated fractions of the pili blendates revealed that the greatest concentration of flexible pili was in the 2% fraction. Therefore, this was re-precipitated with PEG 1% and 2%. Bundles of pili and isolated pili as well as some contaminating flagella fragments were seen in the latter preparation (Fig. 3). Two putative

pilin subunit bands (*c.* 19 kDa and 23 kDa) were obtained on SDS-PAGE analysis of this preparation. These were readily seen on silver-stained gels, but both bands stained poorly with Coomassie Brilliant Blue. However, the 23-kDa band was the predominant protein recovered.

N-terminal amino-acid sequencing and relationship to other purified Aeromonas pilins

N-terminal analysis of this 23-kDa protein was determined on two occasions with two different pili preparations. Unambiguous sequences (13 and 30 amino acids, respectively) were obtained. These showed that the 23-kDa protein was type IV pilin (Fig. 4). Fig. 4 also compares the *A. caviae* pilin sequence with the sequences of other *Aeromonas* type IV pili purified to date. These clearly form a related family of pilins which we have collectively named 'bundle-forming pili' (Bfp) because of their tendency to form bundles on the bacterial surface [21]. Bfp have been shown to be intestinal colonisation factors for *Aeromonas* species [22–26]. The N-terminal sequence of the purified *A. caviae* pilin was identical to that of a

Fig. 3. Electron micrograph of negatively stained purified *A. caviae* CA195 pili. The preparation contains some contaminating flagella (F). Bar, 0.25 μ m.

Bfp isolated earlier from *A. veronii* biovar sobria (strain BC88). However, the two pili are antigenically distinct, as antiserum to the *A. veronii* biovar sobria Bfp did not react with the *A. caviae* pilus. The reported variability in the first amino acid of the Bfp pilins is most likely a reflection of altered migration patterns on HPLC columns of a modified penultimate amino acid which makes definitive identification of this amino acid difficult.

Sequences derived from two recently cloned *Aeromonas* type IV pilin genes are also shown in Fig. 4 [27, 28]. The cloned pilins, designated 'type IV *Aeromonas* pili' (Tap), form a second family of *Aeromonas* type IV pilins with related N-terminal sequences that differ significantly from those of the

Bfp pilins. Tap pili do not appear to be assembled under the standard bacterial growth conditions [28]. A recent study reported that strain CA195 possesses the Tap gene cluster [28].

Discussion

For *A. caviae* to be a gastrointestinal tract pathogen it must be able to colonise the intestinal mucosa. This study has confirmed reports from other geographical regions that clinical strains of *A. caviae* are able to adhere to epithelial cell lines used as models for intestinal cell binding [1, 8–10]. Moreover, this is the first study to show that highly adherent *A. caviae* possess type IV pili of the family shown to be intestinal colonisation factors for strains of *A. veronii* biovar sobria and *A. hydrophila* [22–25].

Because of high background antibody titres to *A. caviae* proteins, this study was unable to produce a polyclonal antiserum in rabbits which would definitively link the purified 23-kDa type IV pilin to the pili seen on the *A. caviae* surface. However, it was the predominant protein band on SDS-PAGE analysis of purified pili preparations and by analogy with an earlier study on the related pilin from a strain of *A. veronii* biovar sobria we postulate that most pili expressed (including the pili bundles) were composed of it. It is possible that the 19-kDa band observed in purified pili preparations was a second pilin type, but insufficient protein was obtained for sequencing. Long, wavy pili of varying widths (4–6 nm and 7–9 nm) were observed occasionally on individual bacteria and in purified preparations (Figs. 2 and 3). The strain from which the 23-kDa Bfp was purified possesses the Tap gene cluster, so it has the potential to produce this second family of type IV pilin also, although in none of the pili purification studies to date has this pilin type been isolated, despite the widespread occurrence of the gene [28].

Purified pilins (Bfp)		Reference
<i>A. caviae</i> (CA195)	mTLIELVIVIIILGILAVTAAPKFLNLQDD	[21]
<i>A. veronii</i> biovar sobria (BC88)	MTLIELVIVIIILGILAVTAAPKFLN	
<i>A. sobria</i> (Ae24)	YTLIELVIVIIILGILADDA	[22]
<i>A. sobria</i> (TAP13)	YTLIELVIVIIILGILA	[23]
<i>A. sobria</i> (Ae1)	MTLIELVIVI	[24]
<i>A. hydrophila</i> (Ae6)	MTLIELVIVI	[25]
<i>A. trota</i> (1220)	YTLIELVIVIIILGILAVTAAPKFL	[26]
Cloned pilins (Tap)		
<i>A. hydrophila</i> (Ah65)	FTLIELMIVVAIVAILAAVALPAYQNYTKK	[27]
<i>A. veronii</i> biovar sobria (BC88)	FTLIELMIVVAIVAILAAI ALPAYQTYTKK	[28]

Fig. 4. The N-terminal amino acid sequence of the purified 23-kDa pilin of *A. caviae* strain CA195 compared with the published sequences of other *Aeromonas* pilins.

Eley *et al.* reported seeing different types of filamentous appendages on *A. caviae* strains and postulated that patterns of cell line adhesion seen after various bacterial growth conditions reflected the expression of different adhesins [11]. They observed that HEP-2 adhesion patterns varied from localised to aggregative in the first phase of exponential growth to a persisting diffuse adherence after the start of stationary phase. Under the assay conditions chosen for the present experiments *A. caviae* strains showed a diffuse adhesive pattern and adhesion to HEP-2 cells was only in part mediated by filamentous structures. Merino *et al.* suggested that lipopolysaccharide is another important adhesin for *Aeromonas* strains binding to HEP-2 cells [29].

The Australian *A. caviae* strains showed little evidence of toxin production either under standard assay conditions or when grown under conditions reported to favour production of toxins by *A. caviae* isolates in the USA [16, 17]. The mechanism(s) by which adherent *A. caviae* may bring about diarrhoeal disease, therefore, remain in doubt. However, this study has identified a pilus marker for strains that are likely to be significant *in vivo*.

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