Epidemiol. Infect. (1994), **112**, 291–298

Characterisation of haemolytic activity from *Aeromonas caviae*

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(*Accepted 7 September 1993*)

SUMMARY

Aeromonas caviae, an enteropathogen associated with gastroenteritis, displays several virulence characteristics. Studies on the kinetics of growth of *A. caviae* and expression of β-haemolytic toxin revealed that *A. caviae* produced maximum haemolytic activity extracellularly during the stationary phase. Preliminary studies on the properties of *A. caviae* haemolysin suggested that divalent cations (Mg^{2+} and Ca^{2+}) and thiol compounds, dithiothreitol and mercaptoethanol enhanced the haemolytic activity. Addition of L-cysteine, glutathione and EDTA reduced the haemolytic activity. The iron chelator, 2-2′ bipyridyl, significantly inhibited the growth of *A. caviae* possibly by iron limitation, with parallel enhancement of haemolysin production compared to *A. caviae* grown in excess of iron. These results suggest that *A. caviae* produces only β-haemolysin, which resembles the haemolysins reported for several other bacteria and the activity might be regulated by environmental factors especially iron.

INTRODUCTION

There is increasing evidence that the enteropathogen *Aeromonas caviae* is a causative agent of gastroenteritis [1–6] and is a predominant isolate among the microbiota of diarrhoeal stool specimens of infants, from intestinal and extra-intestinal infections, and from aquatic ecosystems [4, 7–10]. The regulation and expression of putative microbial virulence factors by the environment and cytoadherence to host cells are essential events in the establishment of pathogenicity. *A. caviae* has various such potential pathogenic attributes. It produces either or both thermostable metalloprotease and a thermolabile serine protease [11, 12]. *A. caviae* has been shown to adhere to host cells [4, 13, 14] and cause structural damage; and even cell death in some cases by displaying cytotoxic factors [4, 6, 15]. *A. caviae* produces an extracellular heat-stable cytotoxin that is lethal to HEp-2 cells [4, 16] and a cytotoxic enterotoxin that exhibits cross-reaction with the cholera toxin [4, 6, 13, 15]. It also encodes either α or β-haemolysin [5, 11, 17] and a haemagglutinin [18, 19]. This study reports the

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preliminary characterization of *A. caviae* β-haemolytic toxin activity and the role of iron in regulating the growth of *A. caviae* and β-haemolysin production.

MATERIALS AND METHODS

Bacterial strain, growth condition and sample preparation

A. caviae NRRL B 966 was obtained from Northern Regional Research Laboratory, Peoria, IL, USA and was routinely grown in Luria broth (LB) or agar plates containing 5% (v/v) sheep red blood cells.

To study the kinetics of growth and haemolysin production, *A. caviae* was grown in a flask containing LB [a final vol. of 100 ml with 5% (v/v) inoculum from an overnight culture] on a shaker (200 rpm) at 37 °C. At various time intervals, samples were collected and their optical density (660 nm) determined. Culture was then centrifuged at 12000 rev./min for 10 min at 4 °C. The culture supernate was then passed through 0·22 μm Millipore membrane filter and the culture filtrate collected and stored at −20 °C. The cells were subjected to osmotic shock [20] to release their contents in order to characterize cell-associated haemolytic and alkaline phosphatase activities. For other haemolytic activity studies, *A. caviae* was grown to an $A_{660} = 1·3$, and processed as described above.

Haemolysis and alkaline phosphatase assays

Sheep red blood cells (sRBC) 1% (v/v) were washed (2–3 times) and resuspended in sodium citrate (0·005 M) and sodium chloride (0·15 M) buffer, pH 7·0. Haemolytic activity was determined by adding fivefold concentrated culture filtrate or osmotically shocked cells (about 15 μg of protein) to 2·0 ml of above buffer containing washed sheep red blood cells and incubated at 37 °C with gentle agitation. Samples were collected at the end of 2 h (maximum lysis) and unlysed erythrocytes were removed by centrifugation at 10000 rev./min for 10 min in a microcentrifuge. Haemoglobin released in the supernatant of the assay mixture was then diluted and optical density measured at A_{540}. Complete (100%) sRBC lysis was obtained by adding 10 μl of 10% SDS (w/v) with 1% sRBC and percent lysis with the supernate samples was calculated in relation to the complete lysis control. One haemolytic unit was expressed as the amount of experimental sample required to release 50% of haemoglobin content of the erythrocytes.

Alkaline phosphatase activity was assayed as described earlier by utilizing Sigma-104 phosphatase substrate [20]. Protein determination was performed by using BCA Protein Assay Reagent (Pierce Chemical Company).

Influence of various agents on the A. caviae *haemolytic activity*

The effects of divalent cations [Mg^{2+} ($MgSO_4$) and Ca^{2+} ($CaCl_2$)], ethylene diamine tetraacetic acid (EDTA), thiol compounds (L-cysteine, dithiotheritol, glutathione and 2-mercaptoethanol), cholesterol and protease inhibitors Na-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and phenyl methyl sulfonyl fluoride (PMSF) on *A. caviae* haemolytic activity were determined by incorporation into the haemolytic assay mixture. The effect of iron on haemolysis was determined by growth ($A_{660} = 0·435$) of *A. caviae* in LB containing haemin, $FeCl_3$ (200 μM each) or an iron chelator, 2-2′ bipyridyl (BPD; 125 μM).

https://doi.org/10.1017/S0950268800057708 Published online by Cambridge University Press

RESULTS

Haemolysin production during the growth of A. caviae

A. caviae NRRL B 966 produced β-haemolysin on LB agar plates that contained 5% sheep red blood cells. In order to characterize this haemolytic activity further, the kinetics of the growth of *A. caviae* and haemolytic toxin production were determined in LB (Fig. 1). Both the culture filtrate and osmotically shocked cells were employed in the haemolysis assay. The haemolytic toxin activity steadily increased in the medium with time parallel to growth and reached a maximum activity in stationary phase. In contrast, the cell associated activity was low (about 4·7% of total activity). Essentially there was no increase in A_{260} absorbing nucleic acid level in the culture filtrate over 0 h experimental samples; however, alkaline phosphatase activity was found both in cells and culture supernate (data not shown). These results indicate that *A. caviae* produces an extracellular β-haemolytic toxin.

Influence of divalent cations and EDTA on the haemolytic activity of A. caviae

The effects of divalent cation addition on *A. caviae* haemolytic activity are shown in Table 1. An 18% relative increase in haemolytic activity was observed in presence of 1 mM Mg^{2+}, which further increased to about 49% at 10 mM. Similarly, 1 mM Ca^{2+} enhanced the haemolytic activity by 47% and further to 59% at 10 mM. Incorporation of EDTA (1 or 10 mM) to the assay mixture significantly reduced the haemolytic activity. These results show that divalent cations are required for the activity of *A. caviae* haemolysin.

Influence of thiol compounds on the haemolytic activity of A. caviae

The influence of various thiol compounds on *A. caviae* haemolytic activity is shown in Table 2. Addition of 0·1 or 1 mM dithiothreitol elevated the haemolytic activity by 48–49%. A similar effect was seen with mercaptoethanol. L-cysteine and glutathione inhibited the haemolytic activity. These results suggest that dithiothreitol and mercaptoethanol are required for the haemolytic of *A. caviae* activity.

Influence of various treatments on the haemolytic activity of A. caviae

The haemolytic activity of *A. caviae* was found to be temperature sensitive. Incubation of the haemolytic assay mixture at 37 °C for 2 h yielded higher activity, whereas haemolytic activity decreased at or above 42 °C. Pre-treatment of the culture filtrate at 60 °C for 15 min or 100 °C for 5 min almost completely destroyed the haemolytic activity (Table 3). Storage of the culture filtrate at −20 °C also resulted in significant loss (by 11·6% after 24 h and 32·7% after 48 h) of haemolytic activity. Proteinase K (2 mg/ml for 2 h at 37 °C) treatment destroyed the haemolytic activity. These results suggest that the haemolytic activity was of *A. caviae* specified by a thermolabile protein.

To determine whether *A. caviae* haemolytic activity due to non-specific proteases, the effects of the protease inhibitors on *A. caviae* haemolytic activity were studied (Table 3). Both TLCK and PMSF enhanced the haemolytic activity of *A. caviae*. This implies that the haemolytic activity of *A. caviae* is not due to

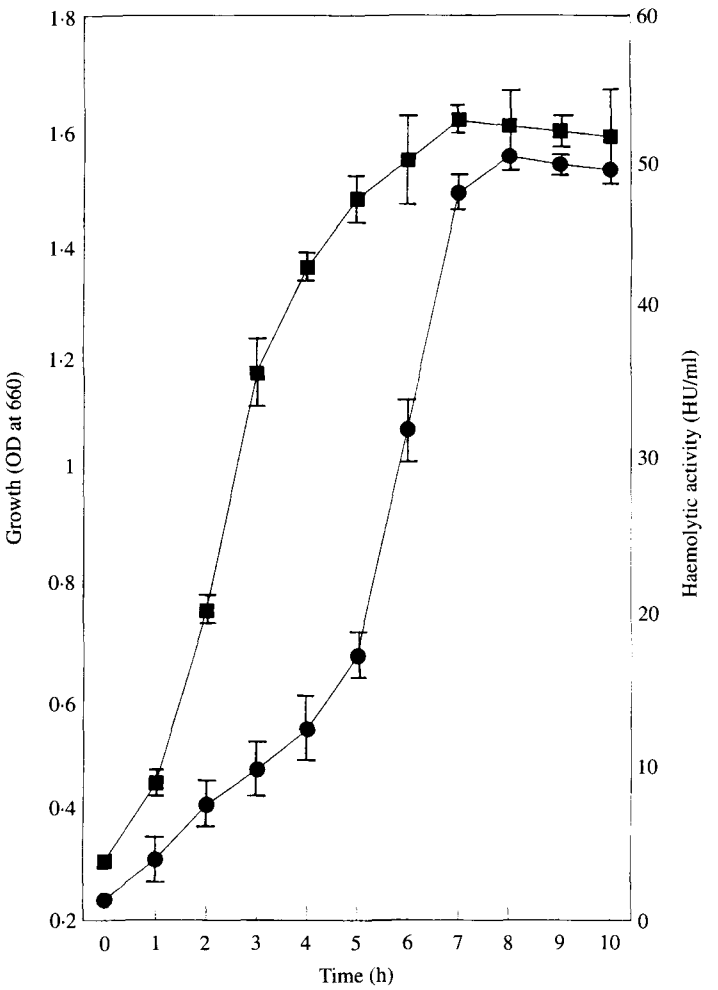

Fig. 1. Kinetics of growth of *A. caviae* NRRL B 966 in LB and extracellular haemolysin toxin production. Flask containing Luria broth was inoculated with 5% (v/v) inoculum of overnight culture and grown in a shaker. At various intervals the growth was monitored and the culture filtrate was utilized for haemolytic assay as described in Materials and Methods. Results represent the mean value ±SD of three different experiments. -■-, Growth; -●-, haemolysis.

Table 1. *Influence of* Mg^{2+}, Ca^{2+} *and EDTA on haemolytic activity of* A. caviae

Various treatments	Concentration	Haemolytic activity (HU/ml)*	Relative activity (%)
None added	—	50·2±1·2	100·0
Mg^{2+}	1 mM	59·3±3·5	118·1
	10 mM	74·9±5·2	149·2
Ca^{2+}	1 mM	73·7±0·8	146·8
	10 mM	79·9±1·2	159·2
EDTA	1 mM	6·3±0·5	12·5
	10 mM	4·1±0·4	8·2

* Haemolytic activity represents the mean value ±SD of three experiments in triplicate.

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Table 2. *Influence of various thiol compounds on haemolytic activity of* A. caviae

Various treatments	Concentration	Haemolytic activity (HU/ml)*	Relative activity (%)
None added	—	50·2 ± 1·2	100·0
Mercaptoethanol	0·1 % (v/v)	52·2 ± 1·2	104·0
	0·3 % (v/v)	54·5 ± 1·6	108·6
	1·0 % (v/v)	63·1 ± 1·0	125·7
Dithiothreitol	0·1 mM	74·5 ± 0·4	148·4
	1·0 mM	75·0 ± 1·0	149·4
Glutathione	1 mM	30·5 ± 3·7	60·8
	10 mM	39·1 ± 0·4	77·9
L-Cysteine	1 mM	51·5 ± 1·7	102·6
	10 mM	25·1 ± 3·0	50·0

* Haemolytic activity represents the mean value ± SD of three experiments in triplicate.

Table 3. *Influence of various treatments on haemolytic activity of* A. caviae

Various treatments	Concentration	Haemolytic activity (HU/ml)*	Relative activity (%)
None added	—	50·2 ± 1·2	100·0
60 °C for 15 min	—	2·4 ± 0·4	4·8
100 °C for 5 min	—	2·0 ± 0·2	4·0
−20 °C for 24 h	—	44·4 ± 1·7	88·4
−20 °C for 48 h	—	33·8 ± 0·8	67·3
Proteinase K	2 mg/ml	3·4 ± 0·3	6·8
TLCK	0·1 mM	64·8 ± 1·6	129·1
	0·5 mM	63·3 ± 1·4	126·1
	1·0 mM	58·6 ± 1·7	116·7
PMSF	0·05 mM	73·5 ± 1·6	146·4
	0·5 mM	65·4 ± 2·5	130·3
Cholesterol	0·1 μg/ml	41·8 ± 0·7	83·3
	0·5 μg/ml	50·7 ± 1·2	101·0

* Haemolytic activity represents the mean value ± SD of three experiments in triplicate.

non-specific proteases. Cholesterol at 0·1 μg/ml reduced the haemolytic activity of *A. caviae* by 17% whereas at 0·5 μg/ml no inhibitory response was seen. It suggests that the *A. caviae* haemolysin might act on phospholipids present on the sRBC membrane.

Regulation of the growth of A. caviae *and haemolysin production by iron*

Addition of iron ($FeCl_3$) or haemin (200 μM) to the culture medium did not affect the growth; however it significantly inhibited the haemolytic activity. At an A_{660} of 0·435, the LB grown culture displayed about 8–9 HU/ml while addition of iron in either form reduced the haemolytic activity to 5·7 ± 1·1 HU/ml. To determine whether the β-haemolytic toxin of *A. caviae* was regulated by iron, BPD was added to LB and the growth of *A. caviae* was monitored. Increasing the concentration of BPD from 200 to 1000 μM in LB significantly inhibited (68–80%) the growth of *A. caviae* (Table 4) possibly by iron limitation. In addition, BPD (125 μM) enhanced the *A. caviae* haemolytic activity (25·4 ± 0·98 HU/ml) by threefold compared to LB grown culture and several fold compared to the cultures grown in excess of iron. These results suggest that iron limitation affected the growth of *A. caviae* with parallel enhancement of haemolysin production.

https://doi.org/10.1017/S0950268800057708 Published online by Cambridge University Press

Table 4. *Influence of iron and 2-2′ bipyridyl (BPD) on the growth of* A. caviae

Various treatments	Concentration	Growth (A_{660})*
LB alone	—	1·336 ± 0·105
LB + $FeCl_3$	200 μM	1·326 ± 0·093
LB + Haemin	200 μM	1·243 ± 0·121
LB + BPD	200 μM	0·435 ± 0·032
	400 μM	0·333 ± 0·047
	600 μM	0·328 ± 0·021
	800 μM	0·319 ± 0·018
	1000 μM	0·274 ± 0·012

* Growth after 16 h of incubation at 37 °C on a shaker (200 rpm). Results represent the mean value ± SD of three different experiments.

DISCUSSION

A. caviae a common isolate of diarrhoeal infants, is capable of exhibiting either α or β-haemolytic activities [5, 11, 17]. Whereas several studies [2, 6, 17] reported the absence of haemolysins and cytotoxins among *A. caviae* strains, the repression of the activities was found to be due to the presence of glucose in growth medium [13]. Singh and Sanyal [6] reported that several non-haemolytic *A. caviae* strains switched to produce haemolysin after consecutive passages through rabbit ileal loops. Similar to β-haemolytic *A. hydrophila* strains [21], several *A. caviae* strains also caused significantly more fluid accumulation than the α- and non-haemolytic isolates [6]. These studies, in addition, emphasized that the β-haemolysin caused significant changes in intestinal permeability and it is more prevalent among *Aeromonas* sp. [2]. Furthermore, partially purified β-haemolysin of *A. sorbia* has been found to be lethal to various eukaryotic cell lines [22].

Studies on the kinetics of growth of *A. caviae* and expression of haemolytic activity, due to a protein molecule, suggest that maximum haemolytic activity (50·7 HU/ml) is produced extracellularly during the stationary phase. Janda [11] also reported an extracellular haemolysin activity in several *A. caviae* strains. Aerolysin of *A. hydrophila* [21, 23], salmolysin of *A. salmonicida* [24] and β-haemolysin of *A. sorbia* [22] are also produced extracellularly during the growth of the bacteria. The β-haemolysin of *A. caviae* resembles that of *Porphyromonas gingivalis* [25] as they are stimulated by both Mg^{2+} and Ca^{2+} ions and inhibited by EDTA whereas an exotoxin of *Proteus vulgaris*, *P. mirabilis* and *P. morganii* [26] and lysolipoid endotoxin of *Pseudomonas pseudomallei* [27] required only Ca^{2+} for their enhanced activity. Although the reducing agents, dithiothreitol and mercaptoethanol enhanced the haemolytic toxin activity of *A. caviae*, cysteine and glutathione inhibited the activity to a certain extent. Aerolysin of *A. hydrophila* [23] salmolysin of *A. samonicida* [24] and β-haemolysin of *P. gingivalis* [25] displayed similar properties.

Although protease inhibitors did not inhibit the haemolytic activity it is possible that the protease inhibitors might have inactivated the proteases present in the culture filtrate, which would otherwise have destroyed the haemolysin and thus enhanced the *A. caviae* haemolytic activity. In *A. salmonicida*, an inactive precursor of haemolysin has been subsequently activated by caseinase [28] and

mutants of *A. salmonicida* that lacked caseinase were found to be devoid of haemolytic activity. It is possible that a similar mechanism might be involved in the activation of haemolysin of *A. caviae*.

The present study has demonstrated that *A. caviae* produced a β-haemolysin, which is similar in properties to haemolysins reported from various *Aeromonas* spp. It has been reported that *A. caviae* strains were found to be serum resistant [18] and several non-haemolytic *A. caviae* strains switch to produce haemolysins after consecutive passages through rabbit ileal loops [18, 19]. The level of free iron available within human body and other animals is far below that required for bacterial growth. Therefore it is possible that such an iron restricted environment could have stimulated the production of haemolysin. The present study demonstrates that BPD significantly inhibits the growth of *A. caviae* possibly by iron limitation. Similar to aerolysin of *A. hydrophila* [29] and salmolysin of *A. salmonicida* [24] the β-haemolysin of *A. caviae* is inhibited by iron compounds; however, BPD enhances the haemolytic activity of *A. caviae* by several fold. This supports the hypothesis that the β-haemolysin of *A. caviae* might be regulated by iron limitation. In conclusion these results together suggest that *A. caviae* presumably produces only β-haemolysin and among various different pathogenic mechanisms displayed by *A. caviae*, the hemolytic toxin production is regulated by iron limitation. Whether iron plays a critical role in the pathogenicity of *A. caviae* is yet to be studied.

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