Formation of Extracellular Haemolysin by *Aeromonas hydrophila* in Relation to Protease and Staphylolytic Enzyme

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**SUMMARY**

*Aeromonas hydrophila* strain 83646 produced extracellular haemolysin in various complex or defined liquid media. The haemolytic activity in all media investigated was proportional to the bacterial dry weight at the end of exponential growth, and there is probably no way to stimulate the production of haemolysin specifically. The haemolytic activity appeared late in the autolytic part of the stationary phase. Inhibition of protein and DNA synthesis by chloramphenicol or nalidixic acid revealed that the haemolysin was synthesized during the late logarithmic phase and released into the medium by lysis. Attempts to release the haemolysin from the bacteria by various disintegration methods were all negative. Cyclic AMP did not affect the synthesis or release of haemolytic activity. The haemolysin is probably bound to some bacterial constituent in the cytoplasm and activated upon lysis. Malate dehydrogenase and protease activity accumulated in the medium simultaneously during this lytic phase, while staphylolytic enzyme was released during the logarithmic phase.

**INTRODUCTION**

In the order Pseudomonadales, according to Bergey's *Manual of Determinative Bacteriology* (1957), many members of the genera *Aeromonas, Vibrio* and *Pseudomonas* produce haemolytic factors (Liu, 1957; C.-E. Nord, T. Wadström & B. Wretlind, unpublished). The formation of a haemolysin from *Pseudomonas aeruginosa* has been studied and most of the activity was reported to be cell bound (Altenbern, 1966). Extracellular haemolytic activities from *Vibrio parahaemolyticus* contained phospholipases and haemolytic proteins with no enzymatic activity (Yanagase et al. 1970; Miwatani, Sakurai, Yoshihara & Takeda, 1972). The formation of a haemolysin from *Aeromonas hydrophila* was studied by Caselitz (1966), who found that most strains produced haemolysin and that the activity accumulated extracellularly in liquid media. The yield was higher in Brain heart infusion broth than in some other complex media. Some strains produced more than one haemolysin.

Recently, Wretlind, Möllby & Wadström (1971) reported on the separation of two haemolytic factors from the culture fluid of *Aeromonas hydrophila* strain 83646 by isoelectric focusing. The two haemolysins had very similar properties and are probably two forms of the same protein (B. Wretlind, R. Möllby & T. Wadström, unpublished). This investigation was undertaken to obtain more information concerning the formation of the haemolytic activity under different physiological conditions, in order to permit production
on a scale large enough for purification and a more extensive characterization. Although many cytolytic proteins from Gram-positive bacteria have been studied in great detail, data concerning the corresponding toxins from Gram-negatives are sparse (Bernheimer, 1970). It is uncertain whether most extracellular toxins and enzymes, which are actively excreted through the wall in Gram-positive species (Pollock, 1962), are excreted by a similar mechanism in Gram-negative bacteria, and it is unclear whether they are always products of autoysis or are released from the periplasmic space when found in the culture medium during growth (Raynaud & Alouf, 1970).

METHODS

**Bacterial strain.** *Aeromonas hydrophila* strain B3646 was obtained from N. Coles, University of Adelaide, Adelaide, Australia. This strain was used for the production of staphylolytic enzyme by Coles, Gilbo & Broad (1969).

**Culture media.** The following media were used: (1) Brain heart infusion broth (BHI) (Difco, Detroit, Michigan, U.S.A.). (2) Nutrient broth (Difco). (3) Trypticase soy broth (TSB) (BBL, Division of BioQuest, Cockeysville, Maryland, U.S.A.). (4) A casein hydrolysate-yeast extract medium (CCY) with β-glycerophosphate and sodium lactate as the carbon sources (Arvidson, Holme & Wadström, 1971). In some experiments glucose was substituted for the β-glycerophosphate and lactate. (5) A minimal medium (Davis & Mingioli, 1950) containing 0.5% (w/v) glucose as carbon source. In some experiments, extra ammonium sulphate (0.03 M) was added to the medium (Table 1). Other carbon sources were also used at a concentration of 5 g/l. Casamino acids (Difco) or yeast extract (Difco) were sometimes added. (6) For studies on the synthesis and release of alkaline phosphatase in relation to the haemolytic activity, a phosphate deficient medium described by Torriani (1968) was used in comparison with the standard media above.

**Cultivation techniques.** Unless otherwise stated *Aeromonas hydrophila* was inoculated from fresh agar slants into indented 1 l Erlenmeyer flasks containing 100 ml medium. Flasks were shaken at a speed of 140 rev./min at 22 °C ± 3 °C for 3 or 4 days. Preliminary experiments showed that lower haemolytic activity and bacterial dry weight were obtained at 37 °C. Some experiments were also performed in a 3 l fermentor (Biotec FL 103, Biotec, Stockholm, Sweden), with culture pH maintained at 7.4 by automatic addition of 2 M-NaOH or 1 M-lactic acid. Impeller speed was 800 rev./min, air flow 3 l/min and temperature 30 °C. In all experiments bacterial growth was monitored either by dry wt or by the determination of extinction value (E<sub>600</sub>), where E<sub>600</sub> = 1 corresponded to a bacterial dry weight of 0.6 mg/ml. There was a linear relationship between optical density and dry wt during the logarithmic phase of growth up to a dry wt of 10 mg/ml.

**Assays.** The haemolytic activity on rabbit red blood cells was assayed in serial twofold dilutions of samples in sodium phosphate buffered saline (pH 7.0) according to Takatsy (1950). Microdiluters of 0.05 ml working volume, pipette droppers (Microtiter, Cooke Engineering Co., Alexandria, Virginia, U.S.A.) and plastic trays (Linbro IS-MRC-96, Linbro Chemical Co., New Haven, Connecticut, U.S.A.) were used to permit serial twofold dilutions of 0.05 ml samples in the cups of the tray. A volume of 0.05 ml of rabbit erythrocytes, suspended in buffered saline to a final concentration of 1% (v/v) was then added to each cup. The trays were read after incubation at 37 °C for 1 h followed by 4 °C for 2 h. One haemolytic unit was defined as the amount of haemolysin in a certain dilution which lysed 50% of the erythrocytes. This micromethod gave one step lower activity than the tube dilution test described earlier (Wadström, 1968).

Alkaline phosphatase (EC. 3.1.3.1) was determined according to Torriani (1968) using
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\(^p\)-nitrophenylphosphate as a substrate. Malate dehydrogenase was determined according to Murphey, Barnaby, Lin & Kaplan (1967). One unit was defined as the amount of enzyme required to cause a change in extinction at 340 nm of 1.00 extinction unit/min, and corresponded to the oxidation of 0.48 \(\mu\)mol of DPNH/min. A standard enzyme preparation with known activity was included for each assay.

Staphylolytic activity was determined according to Coles, Gilbo & Broad (1969). \textit{Staphylococcus aureus} strain Copenhagen heated to 100 °C for 10 min was suspended in 0.05 M tris-glycine buffer, pH 8.5, to an \(E_{660}\) of 0.8. Samples of 0.1 ml were added to 2 ml of the suspension in a water bath at 37 °C. One unit of enzyme activity gave a decrease of 0.001 extinction unit/min in this test system. Protease was measured by the caseinolytic method described by Kunitz (1946) and modified by Arvidson, Holme & Wadström (1971).

Studies on synthesis. Chloramphenicol or nalidixic acid was added to cultures in different phases of growth in order to stop protein synthesis. Chloramphenicol was used at a final concentration of 50 \(\mu\)g/ml and nalidixic acid at 100 \(\mu\)g/ml. For studies with cyclic AMP the following procedure was used. \textit{Aeromonas hydrophila} was cultivated overnight in minimal medium supplemented with 0.25 % (w/v) Casamino acids (Difco). Bacteria were harvested and suspended in the same medium to an \(E_{660}\) of approx. 4 and divided into three flasks. For the induction of \(\beta\)-galactosidase, isopropyl-\(\beta\)-D-thio-galactopyranoside was initially added to a final concentration of 1 mm. Cyclic AMP was dissolved in 0.2 M-sodium phosphate buffer, pH 7.5, and added to two of the three flasks to give final concentrations of 5 mm and 18 mm. The flasks were placed in a shaker at room temperature. Samples were taken at intervals for haemolysin assay and 0.2-ml samples were added to tubes containing two drops of toluene and \(p\)-nitrophenyl-\(\beta\)-D-galactopyranoside (ONPG) at a concentration of 2.66 mm. The tubes were incubated in a water bath at 37 °C for 1 h. Hydrolysis of ONPG was stopped by addition of Na\(_2\)CO\(_3\), 0.25 M final concentration, and the extinction at 420 nm was measured. The whole procedure was previously described in principle by Swenson (1972) for \textit{Escherichia coli}.

Resting bacteria. Strain 83646 was cultivated in CCY\(_1\) medium with 1 % (w/v) glucose as the carbon source in indented flasks as described above. Bacteria were taken from the early and late logarithmic phases and the stationary phase, washed twice in 0.06 M-potassium phosphate buffer, pH 7.0, and then suspended to a density of \(E_{660} = 5\) in minimal medium with no added carbon or nitrogen source. Each suspension was divided between two flasks. Glucose was added to one flask to a final concentration of 0.5 % (w/v). Both flasks were placed on a rotary shaker at 22 °C. Samples were taken after 30 min and every hour for 6 h.

Release experiments. \textit{Aeromonas hydrophila} was cultivated in phosphate deficient medium (Torriani, 1968) and harvested during the exponential or early stationary phase. The bacteria were washed once in 0.04 M-tris-HCl buffer, pH 8.2, then suspended in the same buffer containing 4 mM-ethylenediaminetetraacetic acid (EDTA), 0.5 mM-sucrose and 225 \(\mu\)g egg white lysozyme/ml (Garrard, 1971). The same amount of bacteria was also lysed in a solution containing the same amount of tris-HCl, EDTA and lysozyme without sucrose. Bacteria from the exponential and stationary phases of growth in BHI were disintegrated by freeze pressing (X-press, Biotec) (Edebo, 1960), or by ultrasonic treatment (MSE Sonifier; amplitude 4 to 8 \(\mu\)m, 1 min, 4 °C).

Materials. The following chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A.: Malate dehydrogenase (l-malate: NAD oxidoreductase, EC. 1.1.1.37) from pig heart; trypsin, 2 \(\times\) crystallized; protease from \textit{Streptomyces griseus} (type VI); ribonuclease A (EC. 2.7.7.16) from bovine pancreas (type XII-A); deoxyribonuclease I (EC. 3.1.4.5) from bovine pancreas; egg-white lysozyme, 3 \(\times\) crystallized (EC. 3.2.1.17);
p-nitrophenyl phosphate; isopropyl-β-D-thiogalactopyranoside; and β-diphosphopyridine nucleotide, reduced form (DPNH). ONPG, EDTA, sucrose, lithium chloride and urea of analytical grade were obtained from Merck, Darmstadt, Germany. Adenosine-3’-5’ monophosphoric acid (cyclic AMP) was obtained from Boehringer Mannheim, Mannheim, Germany. Chloramphenicol was purchased from Parke, Davis & Co., Detroit, Michigan, U.S.A., and nalidixic acid from Winthrop Laboratories, Newcastle.

**RESULTS**

**Medium composition.** Maximum haemolytic activity was obtained in the autolytic phase of growth between 36 and 80 h. The optimal biomass of each culture and the haemolytic activity were compared (Table 1). The haemolytic activity seemed to be proportional to the maximum culture turbidity at the beginning of the stationary phase for each medium tested. CCY, medium gave the highest haemolytic titre and bacterial turbidity in cultures with good aeration, but in flasks without indentations maximum culture turbidity was only 1/6 of the aerated control level while the haemolytic activity was negligible. The poor growth was due to lactate and glycerophosphate not being utilized under semi-anaerobic conditions. The degree of aeration was of less importance in BHI, where haemolysin was formed even under anaerobic conditions, since glucose and other compounds are fermented.

**Relationship between formation of haemolysin and other extracellular proteins.** Experiments under defined conditions in a fermentor showed that the staphyloytic enzyme appeared in the medium during the logarithmic phase of growth (Fig. 1). The haemolytic activity...
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Table 1. Relationship between growth and extracellular haemolytic activity

The conditions for cultivation are described in Methods. Culture turbidity (E₆₅₀) was determined at the end of the logarithmic phase of growth and the haemolytic activity 12 to 48 h later.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Maximum biomass (E₆₅₀)</th>
<th>Haemolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM, 0·5% glucose (0·028 M)</td>
<td>2-9</td>
<td>32</td>
</tr>
<tr>
<td>MM, 0·5% glucose, 0·03 M-ammonium sulphate added</td>
<td>3·3</td>
<td>64</td>
</tr>
<tr>
<td>MM, 0·5% glucose, 1% (w/v) YE</td>
<td>8·9</td>
<td>32</td>
</tr>
<tr>
<td>MM, 0·5% glucose, 3% (w/v) CA</td>
<td>16·4</td>
<td>128</td>
</tr>
<tr>
<td>MM, 0·5% glucose, 1% YE, 3% (w/v) CA</td>
<td>28·0</td>
<td>128</td>
</tr>
<tr>
<td>MM, without glucose, 3% (w/v) CA</td>
<td>10·4</td>
<td>128</td>
</tr>
<tr>
<td>MM, 0·5% arabinose (0·003 M)</td>
<td>3·9</td>
<td>64</td>
</tr>
<tr>
<td>MM, 0·5% arginine (0·03 M)</td>
<td>0·7</td>
<td>8</td>
</tr>
<tr>
<td>MM, 0·5% succinic acid (0·042 M)</td>
<td>1·9</td>
<td>16</td>
</tr>
<tr>
<td>BHI</td>
<td>10·2</td>
<td>128</td>
</tr>
<tr>
<td>BHI, flasks without indentations</td>
<td>7·6</td>
<td>64</td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>3·2</td>
<td>64</td>
</tr>
<tr>
<td>TSB</td>
<td>13·9</td>
<td>32</td>
</tr>
<tr>
<td>CCY,</td>
<td>25·0</td>
<td>256</td>
</tr>
<tr>
<td>CCY, flasks without indentations</td>
<td>4·0</td>
<td>4</td>
</tr>
<tr>
<td>Phosphate-deficient medium</td>
<td>7·6</td>
<td>64</td>
</tr>
</tbody>
</table>

MM, Minimal medium; BHI, brain heart infusion broth; TSB, trypticase soy broth; CCY, casein hydrolysate yeast extract medium; YE, yeast extract; CA, Casamino acids.

appeared in the autolytic part of the stationary phase together with protease and malate dehydrogenase activity. Similar results were obtained in shake flasks in all media described in Table 1. In phosphate deficient medium alkaline phosphatase appeared extracellularly in the same manner as the staphylolytic enzyme, but a direct comparison was not possible since staphylolytic enzyme is formed in detectable quantities only in complex media, where the formation of alkaline phosphatase is repressed. Both enzymes are probably located in the periplasmic space of the bacterial cells and released or actively excreted during growth (B. Wretlind, unpublished observations).

Inhibition of protease formation. Liu & Hsieh (1969) showed that ammonium sulphate inhibits formation of extracellular protease in Aeromonas hydrophila and Pseudomonas aeruginosa. Addition of 0·15 M-ammonium sulphate to the BHI medium inhibited the formation of extracellular protease by A. hydrophila strain ~3646 while the formation of haemolysin or staphylolytic enzyme was not affected.

Release of haemolytic activity. To estimate the proportion of bound and extracellular haemolysin, samples of cultures grown in phosphate-deficient medium were treated with lysozyme, sucrose and EDTA in tris buffer to release the periplasmic enzymes without lysing the bacteria. More than 70% of the alkaline phosphatase activity was released through this treatment, but no activity was released with tris and EDTA alone according to the procedure of Garrard (1971) or by osmotic shock according to Neu & Heppel (1965). No detectable haemolysin was released by any of these procedures, nor was any haemolysin found in the lysate of sphaeroplasts. These were obtained by treatment with lysozyme, EDTA and sucrose in tris buffer, and disintegrated by suspending them in distilled water. Bacteria were also lysed with lysozyme and EDTA, freeze-pressing and sonication, but no haemolytic activity was found in any of the lysates. After freeze-pressing, portions of lysates were treated with 0·1 or 1 mg/ml trypsin, protease from Streptomyces griseus, DNase, RNase,
Fig. 2. Relationship between protein synthesis and the appearance of extracellular haemolysin. Cultivation was in flasks with indentations on a rotary shaker. (a) Control; (b) chloramphenicol (Cla) added during the logarithmic phase of growth; (c), (d) Cla added in the stationary phase. For symbols see Fig. 1.

0.5 M-NaCl, 2 M-LiCl, and 4 M-urea, but haemolysin was not activated by any of these treatments. No haemolysin was released in resting suspensions, even after transfer and subsequent incubation in flasks with or without glucose. The results indicate that the haemolysin is probably bound intracellularly as an inactive precursor.

**Relationship between DNA and protein synthesis and appearance of extracellular haemolysin.** Addition of chloramphenicol during the middle of the logarithmic phase to strain B3646 stopped bacterial synthesis, and no extracellular haemolysin appeared during the next 72 h (Fig. 2). When chloramphenicol was added during the late logarithmic or stationary phase, the haemolytic activity reached the same level as the control. The release of haemolysin was accompanied by release of malate dehydrogenase, but there was no relation between the quantities of malate dehydrogenase and haemolysin released. The same experiment was also performed with nalidixic acid with the same result. These experiments showed that the haemolysin is formed during the late logarithmic phase of growth and probably released by lysis.

**Experiments with cyclic AMP.** Addition of cyclic AMP in concentrations from 5 to 18 mM induced β-galactosidase during the first hour in a similar way to that reported for *Escherichia*
Formation of haemolysin by Aeromonas coli (Perlman, de Crombrugghe & Pastan, 1969). However, no difference in the extracellular haemolytic activity was observed. The same experiment was repeated with addition of chloramphenicol to the flasks during the logarithmic phase of growth, before the glucose was depleted. Only trace quantities of haemolysin were released from these flasks during incubation for 36 h, and no significant difference was found between the culture containing cyclic AMP and the control.

**DISCUSSION**

Very few studies have been devoted to the formation of extracellular proteins by Gram-negative bacteria. Finkelstein & LoSpalluto (1970) reported that cholera toxin (choleragen) is probably actively excreted into the culture medium. However, it is generally believed that toxins of Gram-negative bacteria are released when they lyse and that no active excretion of proteins is possible (Raynaud & Alouf, 1970). In several cases the extracellular enzymes produced by Gram-positive bacteria probably correspond to proteins in the periplasmic space of the bacterial envelope of Gram-negative species (Heppel, 1971). To our knowledge, no example of a periplasmic toxin has so far been described.

Our investigation indicated that the haemolytic activity of *Aeromonas hydrophila* strain B3646 is synthesized intracellularly during the logarithmic phase of growth and later released upon lysis. The haemolysin probably exists in the bacteria as an inactive precursor and it is not released or activated by mechanical or chemical disruption of bacteria harvested in the stationary phase of growth. Failure of bacteria treated with proteases or nucleases to release haemolytic activity indicated that these enzymes are not involved in the conversion of a precursor into active haemolysin as occurs, for example, with botulinum toxin, which is activated by a protease upon excretion (DasGupta & Sugiyama, 1972). This conclusion was also supported by the experiments on inhibition of protease formation by ammonium sulphate, since the haemolytic activity was not affected.

Inhibition of protein synthesis by chloramphenicol during different phases of growth showed that the haemolysin is synthesized during the later part of the logarithmic phase. The kinetics of this formation and release were similar to those for enterotoxin B of *Staphylococcus aureus*. This is a secondary metabolite, whereas enterotoxin A is a primary metabolite and actively excreted during the logarithmic phase (Morse, Mah & Dobrogosz, 1969; Morse & Baldwin, 1971). It seems probable that the synthesis of the haemolysin is repressed during the early phase of exponential growth. Cyclic AMP regulates catabolite and transient repression in *Escherichia coli* (Perlman et al. 1969). The lactose operon of *E. coli* is regulated by cyclic AMP, and preliminary experiments confirmed this also for *Aeromonas hydrophila*. However, no effect of cyclic AMP was found on the formation of haemolysin which suggests that some other kind of regulation is involved. A similar observation was described by Tanaka & Iuchi (1971) for an extracellular proteinase from *Vibrio parahaemolyticus* which is repressed by a mechanism similar to that of catabolite repression. Cyclic AMP could not overcome this repression. *Aeromonas hydrophila* produced haemolysin in all media tested and we could not find any way of specifically stimulating the formation of haemolysin in contrast to observations on several toxins from other bacteria. For instance, it is well known that *S. aureus* produces only small quantities of extracellular toxins and enzymes, such as α-toxin, in a defined medium, in spite of good growth, but that yeast extract stimulates the formation of this toxin (Arbuthnott, 1970). Callahan, Ryder & Richardson (1971) found that certain amino acids stimulated the formation of cholera enterotoxin (choleragen) in a chemically defined medium.

Fig. 1 shows that there are two different mechanisms for the release of extracellular
proteins from *Aeromonas hydrophila*. Staphylolytic enzyme was released during growth without lysis. In phosphate deficient medium alkaline phosphatase was released in the same manner. These enzymes are probably periplasmic proteins (B. Wretlind, unpublished data). *Escherichia coli* and most other species of Enterobacteriaceae do not release periplasmic proteins under these conditions. Recently, 'periplasmic leaky' mutants of *E. coli* and *Salmonella typhimurium* have been isolated (Lopes, Gottfried & Rothfield, 1972). These mutants leak ribonuclease I and other periplasmic enzymes in the absence of leakage of intracellular enzymes. The difference between, on the one hand, *E. coli* and *S. typhimurium* wild-types, and on the other hand, mutants of these organisms and *Aeromonas hydrophila* and *Pseudomonas aeruginosa*, probably reflects differences in the structure of the bacterial envelope. The second mechanism of release was dependent on lysis in the stationary phase and was indicated by haemolysin, protease and malate dehydrogenase activity. A similar situation may occur in mutants of *E. coli* which leak an intracellular β-galactosidase due to a defect in the plasma membrane-cell wall complex (Olden & Wilson, 1972).

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