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Expression of Hemolytic Activity by *Plesiomonas shigelloides*

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More than 90% of the *Plesiomonas shigelloides* strains that we tested produced a β-hemolysin, as judged by the results of agar overlay and contact-dependent hemolysis assays. The hemolysin was cell associated, was active against the erythrocytes of various animal species, and was synthesized at both 25 and 35°C. Activity was lost after thermal or proteolytic treatments or after preincubation in the presence of gentamicin; hemolytic activity did not appear to correlate with the previously established 50% lethal doses for seven of these strains. The hemolysin may play a role in iron acquisition in vivo via the lysis of erythrocytes, liberating hemoglobin, or, alternatively, may be involved in gastrointestinal disease.

Plesiomonas shigelloides is a gram-negative, oxidase-positive, facultatively anaerobic organism that currently resides in the family *Vibrionaceae* but whose true taxonomic position is still the subject of controversy (2). The bacterium is most commonly associated with two infectious diseases, namely, gastroenteritis and bacteremia. In patients with diarrheal disease, *P. shigelloides* has been linked to isolated cases of enteritis and dysentery-like syndromes in individuals returning from travel in foreign countries, particularly Mexico, or after the consumption of shellfish or untreated water (7, 10); in several instances, major outbreaks of diarrheal disease in Japan have been attributed to this bacterium (13). In extraintestinal infections, plesiomonads have been shown to cause septicemia in both adults and children; however, their most devastating illnesses have involved cases of neonatal sepsis and meningitis in infants whose mothers have documented histories of complicated deliveries including prolonged membrane rupture (3); for these infants mortality rates exceed 70%.

Although *P. shigelloides* is a reputed enteropathogen, some studies (6), including a recent report on healthy American students in Mexico (9), have failed to document a role for this organism in diarrheal disease. Two major stumbling blocks to conclusively establishing that this microorganism is an enteric pathogen are the lack of an appropriate animal model and the inability to identify a potential virulence mechanism operative in a majority of strains associated with gastrointestinal infections. In 1991, Daskaleros et al. (4) reported that several strains of *P. shigelloides* produced a hemolysin, which appeared to be partially iron regulated and whose expression seemed to be additionally controlled by viscosity and anaerobiosis. In this report, we confirm the initial results of Daskaleros et al. (4) and show that most *P. shigelloides* strains secrete a β-hemolysin which may be the major virulence factor associated with this reputed enteropathogenic species.

MATERIALS AND METHODS

Bacterial strains. Thirty-six strains of *P. shigelloides* recovered from human (intestinal [n = 25], extraintestinal [n = 7]), animal (n = 2), and environmental (n = 2) sources were used in the study described here. The biochemical, serologic, and phenotypic characteristics of a majority of these strains have been described previously (1); strains PS-5 and PS-9 were recovered from aquarium water and human feces, respectively. All strains were judged to be nonhemolytic on routine blood agar plates. Cultures were maintained on extract agar slants at ambient temperatures during the course of the investigation. All assays were performed at 35°C unless otherwise mentioned.

Screening for *P. shigelloides* hemolytic activity. Two methods were used to detect the hemolytic activity present in *P. shigelloides*. In the first procedure, an agar overlay (AO) assay (4) was used. A 3-μl suspension of an overnight tryptone culture of each strain was spotted onto the surface of an L agar (1% tryptone, 0.5% yeast extract, 0.05% NaCl, 1.5% agar) plate and the plate was then incubated for 18 h. After overnight growth, each plate was overlaid with 5 ml of a soft agar suspension (phosphate-buffered saline [PBS] and 0.065% agar) containing 1% washed human type A (HuA) erythrocytes. After allowing the overlay to solidify, plates were reincubated for an additional 4-h period; plates were visually read for signs of hemolysis at hourly intervals during this 4-h period. Selected strains were additionally screened on heart infusion agar for hemolytic activity in the AO assay against guinea pig, sheep, rabbit, and bovine erythrocytes (PML Microbiologicals, Sacramento, Calif.).

In the second procedure, a modification of the contact-dependent hemolytic assay was used (8). Briefly, a 50-μl aliquot of an overnight tryptone culture of each strain was mixed in a 96-well microtiter plate with an equal volume of 2% HuA erythrocytes in PBS. The plates were then incubated at 35°C and were read at hourly intervals for signs of hemolysis. *Escherichia coli* HB101 served as a negative control for both assays.

Iron utilization assays. Selected strains of *P. shigelloides* were evaluated for their ability to use various organic sources of iron (hemoglobin, hemin, hematin, ferritin, protoporphyrin IX) in iron-deficient medium (11). Individual cultures of each strain were seeded at 10^4 CFU/ml into L agar containing 250 μg of defferated ethylenediaminedihydroxyphenylacetic acid (EDDA) per ml. After the agar was poured into the plates and was allowed to solidify and dry, 3-mm-diameter wells were punched into the agar of each plate (up to six per plate) and 15 μl of each solution

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TABLE 1. Hemolytic activity and iron utilization studies on selected *P. shigelloides* strains

Strain no.	Hemolytic activity against erythrocytes of[a]:					Iron utilization of[b]		
	Guinea pig	Sheep	Bovine	Rabbit	HuA	Hemoglobin	Hematin	Hemin
PS-3	−	+	+	−	−	+++	+	+
PS-4	−	+	−	+	−	+++	+	++
PS-5	+	++	−	++	+	+++	+	+
PS-9	++	+++	+++	+++	+	++	+	+
PS-16	+++	+++	−	+++	+++	+++	++	++
1766-81	+++	+++	++	+++	++	+++	+	++
2599-78	++	+++	+	+++	++	+++	++	++

[a] As determined by agar overlay at 1 h; +++, zone diameter, >3 mm; ++, zone diameter, 1 to 3 mm; +, zone diameter, <1 mm; −, no zone detected.
[b] As determined in L agar-EDDA (250 μg/ml); +++, zone diameter, >15 mm; ++, zone diameter, 10 to 15 mm; +, zone diameter, <10 mm.

containing an organic compound was then added to the individual wells. Plates were then incubated overnight, and the zones of bacterial growth surrounding each well were measured (in millimeters). Relative growth in the presence of iron-containing compounds was as follows: +++, >15-mm zone diameter; ++, 10- to 15-mm zone diameter; +, <10-mm zone diameter; −, no zone.

Hemolysis kinetics. *P. shigelloides* PS-16 (from a pelican) and 2599-78 (human blood isolate) were grown in tryptone broth overnight at 35°C. The resultant growth was pelleted and resuspended in PBS to an A_{610} of 0.3, and an equal mixture, by volume, of bacteria (1.0 ml) and 1% (vol/vol) guinea pig erythrocytes in PBS was incubated at 35°C. At specified intervals, an aliquot was removed and centrifuged to remove the erythrocyte debris, and the supernatant was assayed at A_{515} for hemoglobin release. Results were expressed as percent hemolysis relative to the hemolysis of a saponin-lysed control.

RESULTS

We screened 36 strains of *P. shigelloides* for hemolytic activity using two different assays. Of the 36 strains tested, 34 (94%) were positive in one or both assays. By the AO assay, 92% of the *P. shigelloides* strains (n = 33) were hemolytic within 4 h by the AO technique with HuA erythrocytes; 94% of these hemolytic strains were positive within the first hour of incubation and could often be detected in strains in as little as 15 min of incubation. The hemolysis was beta-hemolytic in nature, with clear zones of lysis surrounding individual overlaid colonies. By the contact-dependent assay, only 45% of the strains (n = 15) were judged to be hemolytic within the identical time period, with most strains taking between 2 and 4 h to become positive. Only two strains (PS-12 and PS-34) were found to be negative in both assays; both were fecal strains. None of the 29 strains from traditional enteropathogenic species (*Shigella* spp. [n = 5], *Yersinia enterocolitica* [n = 5], *Salmonella* spp. [n = 5], enteropathogenic *E. coli* [n = 8], and enteroinvasive *E. coli* [n = 6]) nor *E. coli* HB101 was positive in either assay.

On the basis of the results of the preliminary studies described above, we further investigated the activity of this hemolysin and other properties potentially associated with this enzyme in selected strains. The β-hemolysin of *P. shigelloides* was active against a wide variety of erythrocytes from different species, including guinea pigs, rabbits, sheep, and cows (Table 1). The best activity was noted with sheep, rabbit, and guinea pig erythrocytes, while bovine erythrocytes were the least reactive. Since one potential role for the β-hemolysin could conceivably be the in vivo lysis of erythrocytes to release iron-containing hemoglobin for growth, we evaluated the ability of various organic sources of iron to support growth in iron-deficient medium. Of the compounds tested, hemoglobin supported growth best in iron-depleted medium; this was followed by hemin and hematin. Neither ferritin nor protoporphyrin IX (iron-free) permitted growth.

We previously observed (8) that strains of *Edwardsiella tarda* produce a hemolysin that is cell associated under normal conditions and that is not released into the extracellular space upon in vitro culture. We therefore compared the relative hemolytic activity of *E. tarda* ATCC 15947^T with those of two strains of *P. shigelloides* isolated from different geographic sources; both *P. shigelloides* strains were positive in the contact-dependent assay. *E. tarda* ATCC 15947^T caused 100% lysis of guinea pig erythrocytes within 15 min of incubation, while appreciable hemolytic activity in *P. shigelloides* was not detected until between 30 and 45 min of incubation; lysis equivalent to that detected in strain ATCC 15947^T was not observed until between 1 and 2 h postincubation. By using 2599-78 as the prototype strain, hemolytic activity could be substantially reduced (60 to 80%) by preincubation of bacteria with proteases (pronase, trypsin, bromelain, thermolysin), heat inactivation (100°C, 10 min), or coincubation with a high concentration of gentamicin (50 μg/ml).

Finally, we looked at the expression of hemolytic activity at two temperatures. All seven strains of *P. shigelloides* tested demonstrated hemolytic activity at both 25 and 35°C, although slightly greater activity was noted for all strains at the lower temperature. Hemolytic activity, which was based on both growth and hemoglobin release, did not correlate with the 50% lethal doses previously generated for these strains (1).

DISCUSSION

In agreement with the results of Daskaleros et al. (4), we found that >90% of all *P. shigelloides* strains tested produced a protein with apparent beta-hemolytic activity. This protein appears to be cell associated under routine culture conditions and is not released extracellularly in appreciable quantities; it has activity against a wide array of erythrocytes from a number of vertebrate species and can be detected by the AO assay within a few minutes of incubation at 35°C. Unlike Daskaleros et al. (4), we were unable to consistently produce extracellular hemolysin in iron-depleted medium using five different strains and a variety of iron-restricted conditions, although for one strain (PS-9, a fecal isolate), we were able to sporadically release hemolysin with 10 μg of

EDDA per ml in L broth. The reasons why we were unsuccessful are not readily apparent, although in the study of Daskaleros et al. (4), only one of three strains tested produced significant cell-free hemolytic activity under the conditions described for their study. To obtain our results, we had to work with bacterial cultures rather than cell-free supernatants.

The hemolysin has the characteristics of a protein, being inactivated by proteases and high temperatures. Gentamicin, an aminoglycoside, also inhibited activity, suggesting that cellular functions or viability are required for continued enzymatic activity. Our failure to completely inactivate this hemolysin under a variety of conditions may be related to the large concentration of bacteria used in the present study or to a second minor hemolytic component (ca. 20%) that is heat stable. When compared with the hemolysin of *E. tarda*, the hemolysin of *P. shigelloides* was not nearly as active against guinea pig erythrocytes. This result may be related to the number of molecules present on the cell surface, receptor-ligand binding (hemolysin-erythrocyte interaction), or the inherent activity of the enzyme itself. Regardless of the reason, this activity is appreciably weaker than that of *E. tarda* and may be responsible for our inability to detect it consistently in cell-free supernatants.

From the data presented here, it is unlikely that the hemolysin is a major virulence factor in systemic infections since *P. shigelloides* is rarely invasive (2, 3) and the hemolytic activities of selected strains did not correlate with pathogenicity in mice. However, the hemolysin may play one of several roles such as by making iron (hemoglobin) available to the bacterium via erythrocyte lysis or by releasing replicated progeny from the cytoplasm or endocytic vacuoles of invaded cells (12), or alternatively, it may function as an enterotoxin in the gut.

On the basis of the previous studies of Daskaleros et al. (4) and Gardner et al. (5), a variety of enzymatic activities of *P. shigelloides* appear to be iron regulated. In the case of the β-hemolysin of plesiomonads, expression appears to be related to reduced oxygen tension, cellular function, and an intimate contact between this molecule and the erythrocyte membrane. Further studies aimed at the characterization of this hemolysin and its role in disease seem warranted on the basis of its frequency of occurrence in this microbial species.

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