Hemolytic Activity and Invasiveness in Strains of *Proteus penneri*

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Twenty strains of *Proteus penneri* obtained from the Centers for Disease Control, Atlanta, Ga., were tested for their ability to hemolyze sheep and human erythrocytes, a property that is thought to be connected with the invasiveness and virulence of *Proteus* species. In the logarithmic phase of growth, *P. penneri* cultures are hemolytic for such erythrocytes. This ability is comparable to the hemolysis exhibited by nearly 100% of *P. vulgaris* and *P. mirabilis* strains, which is due to the production of a cell-bound hemolytic factor; is demonstrated only in broth cultures; and seems to facilitate the penetration of *P. penneri* and other *Proteus* species into the cells without cytotoxic effects. In contrast, a filterable alpha-hemolysin, which is produced transiently by a very few strains of *P. mirabilis*, was present in 4 of 20 *P. penneri* strains. This property, which is expressed at a high level over a long period, suggests a chromosomal origin. The penetration of an alpha-hemolytic *P. penneri* strain into Vero cells was accompanied by a drastic cytotoxic effect.

The current description of the genus *Proteus* is that described in *Bergey's Manual of Systematic Bacteriology*, vol. 1 (14). Many changes concerning classification and nomenclature have been made in this genus, mainly on the basis of DNA relatedness groupings. DNA relatedness, which is complemented by all other available markers that are useful in modern taxonomy, may suggest the real phylogenetic relationships. The genus *Proteus* consists of four species: *P. vulgaris*, *P. mirabilis*, *P. myxofaciens* (which is of no clinical importance), and *P. penneri* sp. nov.

As early as 1978, Brenner et al. (1) showed in DNA hybridization studies that *P. vulgaris* is a heterogenous species. The subsequent investigation performed by Brenner et al. (1) revealed the existence of three *P. vulgaris* biogroups. Biogroup 1, an indole-, salicin-, and esculin-negative biogroup, was classified as *P. penneri* in 1982 (7). Although the clinical importance of *P. penneri* has not so far been evaluated, a case report revealing the role of one *P. penneri* strain in urinary calculi formation is noteworthy (10).

Besides biochemical activities, some other biological features of *Proteus* rods, including hemolysin production and invasiveness, were studied recently (8, 9, 11, 12).

MATERIALS AND METHODS

Bacterial strains. A total of 20 *P. penneri* strains described by Hickman et al. (7) were kindly provided by P. B. Smith, Hospital Infections Program, Centers for Disease Control, Atlanta, Ga.; *Escherichia coli* CSH55pHlyTn5 was given to us by Z. Szynkiewicz, Department of Veterinary Microbiology, Academy of Agriculture, Warsaw, Poland.

Hemolysis on solid medium. Bacteria were grown on nutrient agar plates (nutrient broth [Biomed, Warsaw, Poland] supplemented with 2% Bacto-Agar [Difco Laboratories, Detroit, Mich.]) with 5% washed human or sheep erythrocytes. The surface of the medium was meticulously dried to inhibit swarming.

Hemolysis in actively growing cultures in fluid media. To 2 ml of nutrient broth (Biomed) were added 0.2 ml of an 18-h broth culture and 0.2 ml of a 5% standardized human or sheep erythrocyte suspension (9). After 3 h of incubation at

37°C, the mixture was centrifuged at 1,500 × *g*, and the amount of hemoglobin released in the supernatants was measured with a spectrophotometer (6D; Coleman) at 540 nm against controls (100% hemolysis of the same amount of erythrocytes solubilized with the aid of saponin; 1 mg per sample).

Filterable hemolysin production assay. Filterable hemolysin activity was measured by the method described by van den Bosch et al. (16). Originally, bacteria were grown in fresh meat extract broth containing 0.2% glucose (16). In the case of *Proteus* species, the precursor for alpha-hemolysin production, which is present in fresh meat, was not needed. Bacteria were grown overnight at 37°C in nutrient broth (Biomed) supplemented with 0.2% glucose (9). This culture was diluted 1:10 in the same medium and grown with gentle agitation for 3 h at 37°C. The supernatants obtained by centrifugation at 4°C were filtered through a membrane filter (pore size, 0.2 μm; Sartorius) and serially diluted in tubes containing 0.5 ml of Tris hydrochloride-buffered saline (pH 7.5). A total of 0.5 ml of 2% sheep erythrocytes, which was suspended in the same buffer, was added to each dilution. After incubation for 3 h at 37°C, the released hemoglobin was measured in supernatants at 540 nm (16). The filterable, extracellular hemolysin was subsequently termed alpha-hemolysin by analogy to the *E. coli* nomenclature (15) and to distinguish it from the nonfilterable, probably cell-bound hemolysin that was revealed only in fluid cultures.

Vero cell invasiveness. The method described by Peerbooms et al. (12) to determine Vero cell invasiveness was slightly modified. Vero cells 135 were grown in tubes as monolayers in minimum essential medium supplemented with 10% inactivated calf medium and antibiotics (100 U of penicillin per ml and 100 μg of streptomycin per ml) at 37°C in an atmosphere of air with 5% CO_2. The invasion test was carried out by the procedure described by Peerbooms et al. (12). In short, the bacteria were incubated with cells for 1, 2, 3, 5, or 7 h and then removed by washing. The extracellular bacteria were killed with 250 μg of gentamicin per ml. The test cells were lysed with 0.01 M $NaHPO_4$–1% Tween 20 (vol/vol)–0.025% (wt/vol) trypsin; intracellular bacteria were counted on agar plates containing 0.1% phenol to inhibit the swarming of *Proteus* bacilli. The total number and viability of tested cells were estimated simultaneously. All experiments were repeated at least twice.

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TABLE 1. Sheep and human erythrocyte hemolysis in actively growing *P. penneri* cultures

Erythrocytes	No. of strains with the following hemolytic activities[a]:			
	100–75%	75–50%	50–25%	25–0%[b]
Sheep	16	3	1	0
Human	6	8	5	1

[a] The results of hemolysis measured at 540 nm were expressed as the percentage of total hemolysis of the same amount of respective erythrocytes.
[b] Hemolysis values under 25% were considered negative.

RESULTS

Hemolysis on solid medium. Of 20 *P. penneri* strains, 19 strains hemolysed human and sheep erythrocytes and gave zones of different intensities. Four *P. penneri* strains produced entirely clear, yellowish zones.

Hemolysis in actively growing fluid cultures. In nutrient broth supplemented with 0.2% glucose, 19 of 20 *P. penneri* strains caused significant hemolysis of sheep erythrocytes; this ability was less marked when human erythrocytes were used (Table 1).

Production of filterable hemolysin. The four *P. penneri* strains (1655-67, 4910-67, 4732-68, and 0541-74) that exhibited the most intensive hemolysis on blood agar produced a filterable hemolysin. The hemolytic activity measured in crude, undiluted, sterile filtrates was expressed as the percentage of total hemolysis of the same amount of erythrocytes. The percentage of hemolysis was 42% for *P. penneri* 1655-67, 72% for *P. penneri* 4910-67, 89% for *P. penneri* 4732-68, and 100% for *P. penneri* 0541-74; alpha-hemolytic *E. coli* CSH55pHlyTn5 showed 65% hemolysis in the same test system. Titration curves were prepared for each of the filtrates. The results obtained with *P. penneri* 0541-47 and *E. coli* are shown in Fig. 1. The prozone-like phenomenon, which was reflected in the titration curve of *E. coli* (16), was absent in *P. penneri*. The *P. penneri* strains that produced alpha-hemolysin were isolated between 1967 and 1974 (7). They were cultivated in our laboratory for 18 months and tested for the production of alpha-hemolysin at 6-month intervals. Alpha-hemolysin activity was retained at the same level, indicating the stability of this characteristic.

Vero cell invasiveness. Alpha-hemolytic *P. penneri* 0541-74 and non-alpha-hemolytic *P. penneri* 0465-75 were tested for the ability to penetrate Vero cells (Fig. 2). There were significant differences in the penetration of Vero cells by the alpha-hemolytic strain compared with that of the non-alpha-hemolytic strain. Maximal penetration of strain 0465-75 occurred at 2 h and continued slowly at a lower level until 7 h, when the experiment was terminated. During this time period the viability of tested cells was constant (96 to 90%), and no cytotoxic effect was evident. The profile of cell penetration of *P. penneri* alpha-hemolytic strain 0541-74 was markedly different; the number of intracellular bacteria increased until the third hour of incubation and then decreased dramatically. At the same time the number of viable test cells decreased to 40%; moreover, microscopic examinations showed that a significant number of Vero cells (ca. 35%) were disrupted and lysed.

DISCUSSION

The frequency of strains belonging to *P. penneri* in different clinical materials seems to be low (7). This study was restricted to 20 strains from the United States because none
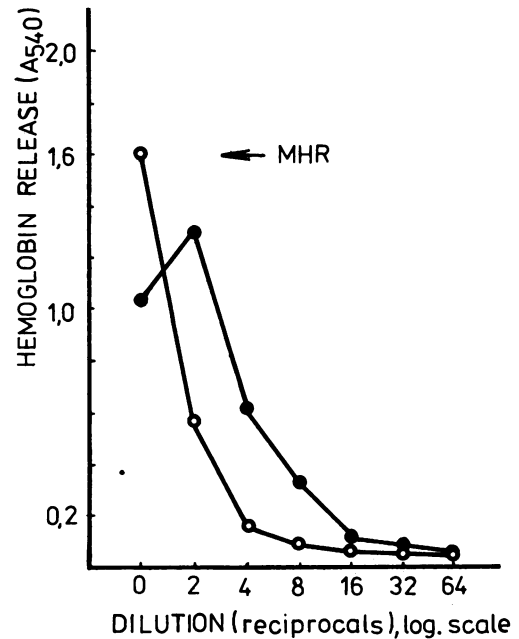

FIG. 1. Alpha-hemolytic activity titrated in filtrates of *P. penneri* 0541-74 and *E. coli* CSH55pHlyTn5 cultures. Abbreviations and symbols: MHR, maximal hemoglobin release; O, *P. penneri*; ●, *E. coli*.

of the 155 clinical isolates of *Proteus* in our collection were *P. penneri* (A. Rozalski, unpublished data).

The ability of *P. penneri* to hemolyze sheep and human erythrocytes on solid blood media was generally comparable to that of other *Proteus* species, hemolyzing erythrocytes with different intensities. All but one of the *P. penneri* strains hemolyzed sheep and human erythrocytes in actively grow-
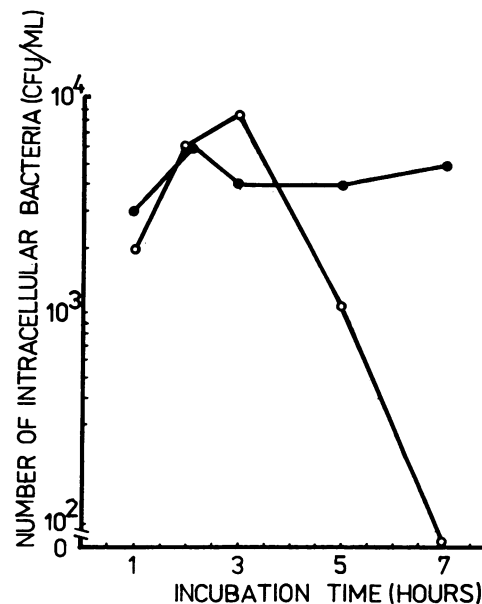

FIG. 2. Vero cell invasiveness of alpha-hemolytic and non-alpha-hemolytic *P. penneri* strains. Symbols: O, alpha-hemolytic *P. penneri* 0541-74; ●, non-alpha-hemolytic *P. penneri* 0465-75.

ing broth cultures. This property is therefore characteristic of the genus *Proteus* (8, 9, 11, 13).

The filterable alpha-hemolysin, which is produced by potentially pathogenic *E. coli* strains (for a review, see reference 6), has also been described in some strains of *Morganella morganii* (formerly *Proteus morganii*) (4) and was also found in three fresh *P. mirabilis* isolates from urinary tract infections (8). They completely lost this property after storage for some months, which indicates that it is mediated by a plasmid. It has recently been shown (3) that alpha-hemolysin activity can be transferred between *E. coli* and *M. morganii* strains both in vivo and in vitro. This supports the suggestion that these two genera produce a hemolysin of common genetic origin (3).

Four *P. penneri* strains persistently produced a filterable alpha-hemolysin, and this ability has been shown to be quite stable for many years. In *P. penneri*, alpha-hemolysin activity may be correlated with cytotoxic activity. This kind of hemolysin is liberated into the environment by many *E. coli* strains (2, 5). The cytotoxic effects of *P. penneri*-soluble hemolysin are different from the action of the common *Proteus* hemolysin that is produced in fluid cultures. The latter seems to facilitate *Proteus* cell penetration without causing cytotoxic effects; moreover, after prolonged observation, survival and multiplication of *P. mirabilis* were shown in stable cell lines as well as in fresh human blood lymphocytes (14a).

ACKNOWLEDGMENT

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