EPITHELIAL CELL ASSOCIATION AND HYDROPHOBICITY OF *YERSINIA ENTEROCOLITICA* AND RELATED SPECIES

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SUMMARY. Six of 11 test strains of *Yersinia enterocolitica* and related species that carried other markers of pathogenicity were found to associate with Henle 407 epithelial cells *in vitro*. All Henle-positive strains were hydrophobic when tested by hydrophobic interaction chromatography with phenyl-Sepharose and by partitioning in an aqueous-hexadecane mixture. Hydrophobicity was also exhibited by some of the Henle-negative strains. None of the test strains aggregated in low concentrations of ammonium sulphate, suggesting that protein structures such as fimbriae were not involved in hydrophobicity or epithelial cell association.

INTRODUCTION

The interaction of *Yersinia enterocolitica* with epithelial-cell cultures *in vitro* was first described by Lee *et al.* (1977) and Une (1977), who observed bacteria within stained monolayers by microscopy. Okamoto *et al.* (1980) used a similar technique and electronmicroscopy to observe adherence of bacteria to the external surface of epithelial cells. Devenish and Schiemann (1981) developed a technique for quantitative expression of infectivity to HeLa cells based on viable cell counts, and related a relative index of infectivity to other virulence indices (Schiemann and Devenish, 1982).

Strains of *Y. enterocolitica* infective for HeLa cells do not exhibit fimbriae by electronmicroscopy (Rahimian and Evans, 1981; Schiemann and Devenish, 1981). Old and Robertson (1981) showed that production of mannose-resistant fimbrial haemagglutinins by *Y. enterocolitica* did not correlate with the ability to adhere to epithelial cells.

Several reports relate the adherence properties of bacteria to hydrophobic characteristics. Smyth *et al.* (1978) reported that hydrophobicity of porcine strains of *Escherichia coli* was related to the presence of the K88 fimbrial adhesin. Wadstrom *et al.* (1980) subsequently showed that hydrophobicity depended on the presence of other fimbrial adhesins on *E. coli*, which in turn determined the ability of the bacteria to adhere to intestinal epithelial cells. Lindahl *et al.* (1981) used aggregation in ammonium sulphate to indicate hydrophobic properties of bacteria, and related this "salting out" to the presence of fimbrial antigens on *E. coli*. Rosenberg *et al.* (1980) described a method based on hydrocarbon adherence for measuring cell-surface

hydrophobicity, which was used to relate adherence to the presence of fimbriae on *Acinetobacter calcoaceticus* (Rosenberg *et al.*, 1981 and 1982) and to smooth and rough forms of *Proteus mirabilis* (Rosenberg *et al.*, 1982).

Two reports are available on hydrophobic properties in *Y. enterocolitica*. Faris *et al.* (1983) observed that mannose-resistant haemagglutination was related to the presence of fimbriae and hydrophobicity as measured by hydrophobic interaction chromatography and aggregation in ammonium sulphate. Lachica and Zink (1984) used binding of cells to polystyrene and xylene and binding of ribosomes to nitrocellulose filters to observe that hydrophobicity was plasmid- and temperature-dependent. In this paper we describe the relationship between hydrophobicity in *Y. enterocolitica* and related species and the ability to associate with Henle 407 intestinal epithelial cells *in vitro*.

MATERIALS AND METHODS

Bacteria. Eleven strains of *Y. enterocolitica* or related species were investigated. All were isolated from human faeces except *Y. enterocolitica* E887, which was isolated from a monkey, and *Y. kristensenii* E812 and *Y. intermedia* E814, which were isolated from pork.

The strain of *P. mirabilis* used as a control culture for measuring hydrophobicity by hydrocarbon partitioning was a clinical isolate obtained from M. Rask, Cedars-Sinai Medical Center, Los Angeles. The K99-positive strain of *E. coli* was a K12 recombinant received from L. Myers, Veterinary Research Laboratory, Montana State University, who also supplied a monoclonal antibody against the K99 fimbrial antigen. Stock strains of all bacteria were stored in peptone-glycerol at -20°C .

Epithelial cell association. Henle 407 intestinal epithelial cells (ATCC CCL-6) were maintained in Eagle's Basal Medium with Hanks's Balanced Salts Solution containing fetal bovine serum 15%, penicillin 50 $\mu\text{g}/\text{ml}$ and streptomycin 50 $\mu\text{g}/\text{ml}$. The cells were grown in tissue-culture flasks until the monolayer was at least 80% confluent. On the day before use, fresh medium without antibiotics was provided. For use, cells were washed twice with Dulbecco's phosphate-buffered saline without calcium (PBS) and harvested by scraping the monolayer off the flask wall. Cell clumps were dispersed by repeated aspiration. The cells were suspended in Eagle's Basal Medium and the number of viable cells was determined microscopically by trypan blue exclusion. The cell density of the suspension was adjusted to $5.6 \times 10^3/\text{ml}$ by dilution with Eagle's Basal Medium.

Bacteria were grown at room temperature for 48 h with constant mixing in a 0.13 M phosphate-buffered salts medium containing: $(\text{NH}_4)_2\text{SO}_4$, 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001%; CaCl_2 , 0.001%; NaCl , 0.1%; yeast extract, 0.1%; and [^{35}S]methionine, 20 $\mu\text{Ci}/\text{ml}$. The medium had a pH of 7.6. Bacteria were harvested by centrifugation at 2800 g for 30 min at 20°C and resuspended in PBS. The bacteria were counted in a Petroff-Hausser chamber and the density was adjusted to $5 \times 10^8/\text{ml}$ with PBS. The adjusted suspension (0.2 ml) was added to 1.8 ml of the Henle-cell suspension. A plate count of the bacterial suspension was performed to determine the number of bacteria per Henle cell.

The mixture was centrifuged at 920 g for 10 min at 20°C and then incubated at 35°C for 30 min. The pellet containing cells and bacteria was gently resuspended and the contents of the tube (2.0 ml) were added to 25–30 ml of PBS on a 5- μm Nucleopore membrane that had been pre-treated with 20–25 ml of 1 mM ethylene glycol-bis- $(\beta$ -amino-ethyl ether) N , N '-tetraacetic acid (EGTA) in 2% peptone (pH 8.3). After filtration under gravity an additional 100 ml of PBS was added to rinse the membrane. Each test was performed in triplicate.

Control suspensions containing bacteria alone were prepared and filtered in a similar manner. The radioactivity of the bacteria was determined by filtering the suspensions through a 0.4- μm Nucleopore membrane. The background reading for broth was determined by centrifuging the suspension at 3700 g for 30 min and filtering the supernate.

Membranes were placed in scintillation vials with 10 ml of Aquasol (New England Nuclear, Boston, MA) and radioactivity was determined by scintillation counting (Tri-Carb 460 CD

Liquid Scintillation System, Packard Instrument Co., Inc., Downers Grove, IL). Counts were converted to disintegrations/min by reference to an efficiency curve based on ^{14}C . The radioactivity/bacterial cell was calculated and this volume was used to determine the average number of bacteria associated with each Henle cell.

Hydrophobic interaction chromatography. Cultures of *Yersinia* spp. were grown as described above. Bacteria were harvested by centrifugation and resuspended in 4 M NaCl in 10 mM phosphate buffer (pH 7.2) to achieve a concentration of $c. 10^9$ cells/ml. *E. coli* was grown on Minca agar (Guinee *et al.*, 1977) at 35°C for 48 h. Bacteria were scraped off the agar and suspended in 4 M NaCl to achieve a concentration of $c. 10^9$ cells/ml.

Sepharose CL-48 and phenyl-Sepharose CL-48 (Pharmacia, Inc., Piscataway, NJ) were washed five times with equal volumes of 4 M NaCl. A volume was added to a Pasteur pipette plugged with glass wool to provide a column of 20–25 mm after settling. The pipette was filled to the top with 4 M NaCl and allowed to drain. The bacterial suspension (100 μl) was layered on the top of the column and 5 ml of 4 M NaCl were added for elution. The number of bacteria added to the column and the number recovered in the eluate were determined by plate counts and the percentage adherent to the column was calculated.

Partitioning in an aqueous-hydrocarbon mixture. Cultures of *Yersinia* spp. were grown as described above. Bacteria were harvested by centrifugation and washed twice with 0.85% NaCl. The density of each bacterial suspension was adjusted photometrically to an absorbance of 1.2–1.4 at 400 nm (DMS 80 Spectrophotometer, Varian Associates, Mulgrave, Victoria, Australia). A control culture of *P. mirabilis* was grown on Trypticase Soy Agar supplemented with yeast extract 0.6% at 35°C for 24 h. Bacteria were washed from the agar, centrifuged and washed twice with 0.85% NaCl.

Four ml of the bacterial suspension were added to an acid-washed glass test tube and the hydrocarbon (xylenes or hexadecane) was added. The tube contents were mixed on a vortex mixer for 1 min and then allowed to stand for 15 min. The aqueous phase was removed from the tube and added to a cuvette. Results were expressed as percentage decrease in absorbance of the aqueous phase at 400 nm.

Aggregation in ammonium sulphate. Cultures of *Yersinia* spp. and a control culture of *E. coli* were grown as described. Bacteria were harvested and suspended in 0.002 M phosphate buffer (pH 6.8) to achieve a concentration of $c. 10^9$ cells/ml. Twenty-five μl of the suspension were added to 50 μl of $(\text{NH}_4)_2\text{SO}_4$ solution in 0.002 M phosphate buffer in a well slide. The concentrations of $(\text{NH}_4)_2\text{SO}_4$ started at 0.02 M and increased in 0.02 M increments to 0.20 M, and then in 0.20 M increments to 2.0 M. The slide was rocked for 2 min and aggregation was observed under a stereomicroscope.

RESULTS

Epithelial cell association

Six of the 11 strains of *Yersinia* exhibited a positive association with Henle epithelial cells (table). An average of 152 bacteria per Henle cell was observed with one strain and an average of 53 (± 14) bacteria per Henle cell with the other five Henle-positive strains. To confirm that the increase in radioactivity observed in the presence of Henle cells was due to associated bacteria, an experiment was designed in which the Henle cells were first exposed to unlabelled bacteria (*Y. enterocolitica* E736) then to radiolabelled bacteria of the same strain. In this case the radioactivity did not exceed the background reading observed with bacteria alone.

All strains of *Y. enterocolitica* exhibiting Henle-cell association also exhibited autoagglutination (Laird and Cavanaugh, 1980) and some other markers of virulence (Devenish and Schiemann, 1981; Schiemann *et al.*, 1981; Karmali *et al.*, 1982; Schiemann and Devenish, 1982). These strains behaved as *Y. enterocolitica sensu stricto* (Bercovier *et al.*, 1980) in biochemical tests. Two of the Henle-negative strains

were identified as *Y. kristensenii* and one as *Y. intermedia*. The remaining two Henle-negative strains were, unlike the Henle-positive strains, able to hydrolyse salicin at 35°C and aesculin at 22°C (Schiemann and Devenish, 1982).

Hydrophobic interaction chromatography

The *E. coli* K99 control culture showed 96% adsorption to phenyl-Sepharose, which was significantly higher than the 60% adsorption to Sepharose alone (table). Four of the 11 strains of *Yersinia* showed >90% adsorption to phenyl-Sepharose, a level often taken to indicate hydrophobicity. For nine of the test strains, adsorption to phenyl-Sepharose was significantly higher than to Sepharose alone. Four of the six Henle-positive strains showed >90% adsorption. Adsorption to phenyl-Sepharose and Sepharose was significantly different for the remaining two Henle-positive strains. Three of the five Henle-negative strains also showed differences in adsorption to phenyl-Sepharose and Sepharose. The difference observed with *Y. kristensenii* E709 was not statistically significant because of the low precision (SD=22.6) of the Sepharose results. There was no difference in adsorption to phenyl-Sepharose and Sepharose by *Y. intermedia* E814. Strains of *Yersinia* adhering to phenyl-Sepharose by

TABLE
Relationship of Henle-cell association to hydrophobicity in *Yersinia* spp.

Test strain (serotype)	Number of bacteria/ Henle cell (SD)	Percentage adsorption (SD)		Decrease in absorbance* (%)	Lowest molarity of (NH ₄) ₂ SO ₄ showing cell aggregation
		Phenyl-Sepharose	Sepharose		
<i>Y. enterocolitica</i> E641 (O:5,27)	152 (±42)	97 (±0.8)	73 (±9.3)†	95 (±0.6)	1.4
<i>Y. enterocolitica</i> E661 (O:8)	46 (±2.9)	87 (±5.8)	1.2 (±2.1)†	69 (±3.5)	1.4
<i>Y. enterocolitica</i> E663 (O:8)	55 (±8.9)	93 (±2.4)	21 (±15.2)†	58 (±6.8)	1.4
<i>Y. enterocolitica</i> E736 (O:21)	37 (±11.5)	82 (±5.4)	48 (±7.2)†	78 (±2.2)	1.4
<i>Y. enterocolitica</i> E739 (O:3)	50 (±20.2)	94 (±1.1)	40 (±7.2)†	60 (±4.4)	NA
<i>Y. enterocolitica</i> E887 (O:13)	75 (±29.7)	93 (±3.8)	23 (±20.8)†	88 (±2.7)	2.0
<i>Y. enterocolitica</i> E668 (O:6,30)	2 (±3.6)	84 (±11.1)	39 (±11.4)†	51 (±5.8)	1.2
<i>Y. kristensenii</i> E709 (O:12,26)	0	89 (±3.3)	54 (±22.6)	62 (±6.0)	1.2
<i>Y. enterocolitica</i> E770 (O:5)	0	65 (±4.3)	25 (±2.2)†	6 (±5.0)	1.2
<i>Y. kristensenii</i> E812	0	70 (±5.0)	2 (±3.1)†	21 (±11.1)	NA
<i>Y. intermedia</i> E814	0	3 (±4.4)	7 (±11.8)	6 (±4.8)	NA
<i>E. coli</i>	NT	96 (±0.3)	60 (±1.3)†	NT	0.02
<i>P. mirabilis</i>	NT	NT	NT	78 (±2.5)	NT

NA = no aggregation observed in 2.0 M ammonium sulphate. NT = not tested.

* Decrease in absorbance at 400 nm of aqueous phase after separation of a mixture of bacterial suspension 4.0 ml and hexadecane 0.5 ml.

† Difference between phenyl-Sepharose and Sepharose statistically significant by Student's *t* test for independent means (*p*=0.05).

the column technique did not do so when bacteria and phenyl-Sepharose were mixed in a vial and allowed to settle. Under these conditions, there was no difference in numbers of bacteria in supernatant fluids from phenyl-Sepharose, Sepharose and a buffer control.

Hydrocarbon partitioning

A preliminary experiment was performed to determine the influence of hydrocarbon concentration and type of hydrocarbon on partitioning in an aqueous-hydrocarbon system. *P. mirabilis* was selected as a control organism because a previous report indicated that it was hydrophobic when tested by this technique (Rosenberg *et al.*, 1982). The results showed that the degree of partitioning of *P. mirabilis* was greater with xylenes than with hexadecane, and was related to the volume of hydrocarbon added (fig.). In contrast, *Y. enterocolitica* E641 (Henle-positive) showed a high degree of partitioning that was unrelated to type or volume of hydrocarbon. For the remaining tests, 0.5 ml of hexadecane was used with 4.0 ml of bacterial suspension.

The control culture of *P. mirabilis* showed 78% decrease in absorbance of the aqueous phase (table). Only three strains of *Y. enterocolitica* (all Henle-positive) exhibited a decrease in absorbance $\geq 78\%$. Three Henle-negative strains showed a very

FIG.—Partitioning of bacterial cells in mixtures of saline and hexadecane (●) or saline and xylenes (○) expressed as percentage change in absorbance of the aqueous phase at 400 nm. Arithmetic mean and range for three determinations at each solvent volume are shown.

low degree of partitioning. The remaining three Henle-positive strains and two Henle-negative strains exhibited similar decreases in absorbance.

Aggregation in ammonium sulphate

The culture of *E. coli* exhibited aggregation in 0.02 M ammonium sulphate, whereas the lowest concentration of ammonium sulphate in which any strain of *Yersinia* showed aggregation was 1.2 M (table).

DISCUSSION

Previous studies established that pathogenic strains of *Y. enterocolitica* can be distinguished by their ability to associate with epithelial cells *in vitro* (Lee *et al.*, 1977; Une, 1977; Okamoto *et al.*, 1980; Devenish and Schiemann, 1981; Schiemann *et al.*, 1981; Schiemann and Devenish, 1982). In this study, strains of *Y. enterocolitica* displaying other markers of virulence associated with Henle 407 epithelial cells, whereas strains lacking these markers did not.

Several reports suggest, mostly by use of semi-quantitative criteria, that epithelial cell association is related to bacterial cell hydrophobicity, and that hydrophobicity is in turn dependent on the presence of fimbriae (Smyth *et al.*, 1978; Wadstrom *et al.*, 1980; Lindhal *et al.*, 1981; Rosenberg *et al.*, 1981; Rosenberg *et al.*, 1982; Faris *et al.*, 1983). Using hydrophobic interaction chromatography to measure hydrophobicity, we had difficulty in interpreting quantitative results. Henle-positive strains of *Yersinia* could be differentiated from Henle-negative strains neither by applying a criterion based on a certain level of adsorption to phenyl-Sepharose, nor by the difference between adsorption to substituted and unsubstituted gels. Although all Henle-positive strains were hydrophobic by one or other of these criteria, so were four of the five Henle-negative strains.

When hydrophobicity was measured by the degree of partitioning in an aqueous-hexadecane mixture, a clearer relationship to Henle-cell association was observed. Three of the five Henle-negative strains were non-hydrophobic and all Henle-positive strains were hydrophobic by this technique. However, two Henle-negative strains were as hydrophobic as Henle-positive strains. Tests of aggregation in ammonium sulphate indicated that the hydrophobicity observed in *Yersinia* spp. by the other two techniques was not related to the presence of protein structures as suggested by other workers (Lindahl *et al.*, 1981; Faris *et al.*, 1983). Hydrophobicity can be conferred by outer-membrane structures other than fimbrial proteins, such as lipopolysaccharides, phospholipids, lipoproteins and outer-membrane proteins.

Strains of *Y. enterocolitica* that were capable of association with Henle 407 epithelial cells were also hydrophobic. However, the converse was not true. This indicates that other factors are necessary for the association of *Y. enterocolitica* with epithelial cells.

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