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Rodney A. Moxley  
*University of Nebraska - Lincoln*, rmoxley1@unl.edu

Emil M. Berberov  
*University of Nebraska - Lincoln*

David H. Francis  
*South Dakota State University, Brookings, South Dakota*

Jun Xing  
*University of Nebraska - Lincoln*

Mahtab Moayeri  
*University of Wisconsin, Madison, Wisconsin*

See next page for additional authors

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Authors
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Pathogenicity of an Enterotoxigenic Escherichia coli Hemolysin (hlyA) Mutant in Gnotobiotic Piglets†

RODNEY A. MOXLEY,1,* EMIL M. BERBEROV,1 DAVID H. FRANCIS,2 JUN XING,3‡ MAHTAB MOAYERI,1 RODNEY A. WELCH,3 DIANE R. BAKER,2 AND RAUL G. BARLETTA1

Departments of Veterinary and Biomedical Sciences, University of Nebraska—Lincoln, Lincoln, Nebraska 68583;1 Department of Veterinary Science, South Dakota State University, Brookings, South Dakota 57007;2 and Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, Wisconsin 537063

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Pigs infected with hemolytic F4+ strains of enterotoxigenic Escherichia coli often develop septicemia secondary to intestinal infection. We tested the hypothesis that inactivation of hemolysin would reduce the ability of F4+ enterotoxigenic E. coli to cause septicemia in swine following oral inoculation. Inactivation of the hemolysin structural gene (hlyA) did not decrease the incidence of septicemia in the gnotobiotic piglet model.

Escherichia coli is an important cause of diarrheal disease and extraintestinal infections in animals and humans (26). Strains that produce enterotoxins (enterotoxigenic E. coli [ETEC]) are among the most important causes of diarrhea in neonatal swine and cattle (20, 45) and are a common cause of septicemia in preweaned swine (8). E. coli bacteria that cause septicaemia express virulence determinants that mediate their spread from the intestine to extraintestinal sites and that mediate bacterial persistence and proliferation in these locations (8). Putative virulence determinants include fimbrae, polysaccharide capsules, O-antigen capsules, lipopolysaccharide, aerobactin, hemolysin, and other cytotoxins (8). P fimbrae and hemolysin are the most important virulence factors that distinguish bacteremia-causing isolates from fecal control strains in human diabetic patients (3). The introduction of hemolysin genes into E. coli bacteria increases the virulence of these strains when inoculated into the peritoneal cavity or bloodstream in laboratory rodents (7, 33, 42). Hypothetically, hemolysin might increase virulence by increasing the availability of iron in the absence of aerobactin (27), mediating toxic effects on leukocytes and other nucleated cells (43), potentiating the effects of endotoxin (43), and mediating serum resistance (31). However, despite evidence suggesting that hemolysin increases the virulence of E. coli, there is little information regarding its role in vivo (43).

It has been shown that hemolytic ETEC strains naturally cause extraintestinal disease in preweaned and weaned swine, manifested clinically by sudden death and pathologically by lesions caused by endotoxic shock (9, 23). These strains most often belong to serogroups O8, O149, and O157 and are gene probe positive for hemolysin, K88 (F4) fimbrae, heat-labile enterotoxin I (LT-I), and heat-stable enterotoxin b (STb) (9, 23, 24). Experimental inoculation of gnotobiotic piglets with hemolytic strains of E. coli causes bacteremia and lesions identical to those seen in naturally occurring cases of hemolytic-ETEC-induced endotoxic shock (9, 14, 16, 23, 24).

The results of these and other experiments using animal models and the association of hemolytic E. coli with extraintestinal disease in swine led us to hypothesize that inactivation of hemolysin would decrease the ability of F4+ enterotoxigenic E. coli to cause septicemia in swine following oral inoculation. Inactivation of the hemolysin structural gene (hlyA) did not decrease the incidence of septicemia in the gnotobiotic piglet model.

Genetic and recombinant DNA procedures. Recombinant DNA procedures were done as described by Sambrook et al. (29). Total genomic DNA was extracted by a guanidium isothiocyanate-EDTA-Sarkosyl lysis procedure, digested with PvuII, and separated by 0.75% agarose gel electrophoresis in Tris-acetate-EDTA buffer (29). Gene probes for hlyCABD and the Km marker were prepared from the 7.0-kb AvaI-A fragment of pWAM04 (44) and from the 1.2-kb BamHI-B fragment of pUC4K, respectively. Gene probes for LT-I and STb were prepared from the 850-bp HindIII fragment of plasmid pDAS102 and from the 460-bp HindII fragment of plasmid pDAS103, respectively (4, 13, 22, 36). The probes were directly labeled with horseradish peroxidase, and the hybridization products were detected with a chemiluminescence detection system (ECL direct nucleic acid labeling and detection system; Amersham International, Little Chalfont, Buckinghamshire, England).

‡ Present address: Department of Internal Medicine, Division of Rheumatology, University of Michigan Medical Center, Ann Arbor, MI 48109.

* Corresponding author. Mailing address: 111 VBS, University of Nebraska—Lincoln, Fair St. and East Campus Loop, Lincoln, NE 68583-0905. Phone: (402) 472-8460. Fax: (402) 472-9690. E-mail: vets044@unlvm.unl.edu.

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Construction of hlyA deletion mutant. A nonhemolytic mutant of strain 2534-86 was constructed as follows (Fig. 1). Plasmid pWAM1097 contains hlyCA from E. coli urinary tract isolate J96, cloned in pUC19 (39). Plasmid pWAM1661 was constructed by removing PmeI-NaeI(hlyA2306–3197) in pWAM1097 and replacing it with a Km r cassette (aphA) from pUC4K (38, 39). This hlyCA region containing the Km r cassette was cloned into the XbaI site of pCVD442 and transformed into S17-1pir to yield donor strain WAM1746. WAM1746 was mated to a spontaneous nalidixic acid-resistant mutant of strain 2534-86, designated WAM2317. Bacterial matings were done at 37°C for 9 to 12 h on 0.45-μm-pore-size filters on Luria-Bertani (LB) agar containing 100 mM sodium citrate. Mating mixtures were subcultured overnight at 37°C on blood agar plates containing nalidixic acid (50 μg/ml) and kanamycin (50 μg/ml) (BA-NAL-KM plates). Five transconjugant colonies with decreased hemolytic zones on BA-NAL-KM plates were selected from the mating and pooled into one culture. This culture was incubated with aeration at 37°C for 5 h, pelleted, and plated for counterselection on LB agar plates containing 5% sucrose, nalidixic acid, kanamycin, and no NaCl (LB-NAL-KM-sucrose). After a 16-h incubation at 28°C, isolated colonies on the LB-NAL-KM-sucrose plates were patched onto LB plates containing 100 μg of ampicillin per ml and onto BA-NAL-KM plates. One nonhemolytic Ap r colony, designated WAM2335, was selected for further examination. A liquid hemolysis assay (2) with dilutions made in 20 mM CaCl2 detected no hemolytic activity in culture supernatant fluids of WAM2335, whereas the peak activity of WAM2317 was approximately 200 50% lysis hemolytic units/ml.

Southern blot analyses of PvuII digests of WAM2317 genomic DNA probed for hlyCABD contained hybridizable fragments of 8.5 and 6.2 kb (Fig. 2A). In contrast, digests of WAM2335 probed for hlyCABD contained hybridizable fragments of 6.2, 6.0, and 3.0 kb (Fig. 2A). The 3.0-kb fragment also hybridized to the aphA gene probe (Fig. 2B). Southern blot analyses of HindIII digests of WAM2335 genomic DNA contained hybridizable bands for LT-I and STb. Expression of F4 fimbrial antigen in WAM2335 was demonstrated by an indirect immunofluorescence assay (data not shown).

Gnotobiotic piglet experiments. Seven gnotobiotic piglets from one litter were derived by closed hysterotomy and reared in sterile isolator units according to standard procedures (18). When the piglets were 8 days old, swab samples from the nasal cavity and rectum and blood samples from the orbital sinus were obtained from each piglet under anesthesia (20 mg of tiletamine hydrochloride-zolazepam hydrochloride [Telazol; Fort Dodge Labs, Fort Dodge, Iowa] per kg of body weight [12, 40]). When the piglets were 9 days old, four were inoculated orally with WAM2335 and three were inoculated orally with WAM2317. Piglets in both groups were inoculated by instilling 1.0 × 109 CFU into the milk replacer. After inoculation, the piglets were examined every 2 to 4 h for decreased rectal temperature, anorexia, vomiting, diarrhea, depression, and a moribund condition. Pre- and postinoculation blood samples, the latter when piglets were moribund or at 65 h postinoculation, were collected in sterile blood collection tubes for bacterial culture.

Rectal and nasal swab specimens and ileal, liver, and lung in sterile isolator units according to standard procedures (18). When the piglets were 8 days old, swab samples from the nasal cavity and rectum and blood samples from the orbital sinus were obtained from each piglet under anesthesia (20 mg of tiletamine hydrochloride-zolazepam hydrochloride [Telazol; Fort Dodge Labs, Fort Dodge, Iowa] per kg of body weight [12, 40]). When the piglets were 9 days old, four were inoculated orally with WAM2335 and three were inoculated orally with WAM2317. Piglets in both groups were inoculated by instilling 1.0 × 109 CFU into the milk replacer. After inoculation, the piglets were examined every 2 to 4 h for decreased rectal temperature, anorexia, vomiting, diarrhea, depression, and a moribund condition. Pre- and postinoculation blood samples, the latter when piglets were moribund or at 65 h postinoculation, were collected in sterile blood collection tubes for bacterial culture.

Rectal and nasal swab specimens and ileal, liver, and lung

### TABLE 1. E. coli strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Vector</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2534-86</td>
<td>O8:K87:NM:F4ac, HlyA r</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>S17-1pir</td>
<td>LT-I r STb r</td>
<td></td>
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</tr>
<tr>
<td>WAM1808</td>
<td>K12</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>WAM2327</td>
<td>Nal r mutant of 2534-86</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>WAM2325</td>
<td>ΔhlyA mutant of WAM2317</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCVD442</td>
<td>MobRP4 r R6K r SacB r Ap r</td>
<td>pGP704</td>
<td>6</td>
</tr>
<tr>
<td>pWAM04</td>
<td>HlyCABD r Ap r</td>
<td>pUC19</td>
<td>44</td>
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<tr>
<td>pUC4K</td>
<td>Km r Ap r</td>
<td>pUC7</td>
<td>38</td>
</tr>
<tr>
<td>pWAM1097</td>
<td>HlyCA r Ap r</td>
<td>pUC19</td>
<td>39</td>
</tr>
<tr>
<td>pWAM1661</td>
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<td>pUC19</td>
<td>39</td>
</tr>
<tr>
<td>pWAM1746</td>
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<td>pCVD442</td>
<td>This study</td>
</tr>
<tr>
<td>pDAS102</td>
<td>LT-I r Ap r</td>
<td>pUC8</td>
<td>4</td>
</tr>
<tr>
<td>pDAS103</td>
<td>STb r Ap r</td>
<td>pUC8</td>
<td>13</td>
</tr>
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specimens were cultured aerobically and anaerobically by standard procedures on sheep blood agar (5% sheep blood in Trypticase soy agar; Remel Labs, Lenexa, Kans.). Blood samples were cultured aerobically and anaerobically in commercial brain heart infusion broth culture vials (Septi-Check; BBL Labs, Cockeysville, Md.). Bacterial isolates were identified by standard procedures. Clinical parameters and mean colony counts per gram of ileal mucosa were compared by using Student’s t test (34).

All piglets became depressed, anorectic, and diarrheic after inoculation. All piglets developed diarrhea by 22 h postinoculation, and it persisted until euthanasia or death (38 to 65 h postinoculation). Three of four piglets inoculated with WAM2335 and one of three piglets inoculated with WAM2317 became hypothermic and moribund at 36 to 42 h postinoculation (P > 0.10). Cultures of preinoculation rectal and nasal specimens from three piglets inoculated with WAM2335 and one piglet inoculated with WAM2317 yielded Staphylococcus spp. The preinoculation blood samples from all piglets were sterile. Cultures of the ileal specimens collected at necropsy yielded the respective E. coli inoculum strain from each of the piglets. Mean CFUs of WAM2335 and WAM2317 per gram of ileal mucosa were not significantly different (P > 0.05). WAM2335 was isolated from the liver, lung, and blood of three of four inoculated piglets. WAM2317 was isolated from the lungs of the three inoculated piglets but from the liver and blood of only one of these piglets (P > 0.10).

All seven piglets were determined to be of F4 adherence phenotype A (i.e., susceptible to F4ab, F4ac, and F4ad organisms) in the brush border adherence assay (1). Ultrastructurally, WAM2335 organisms were adhered to the apical surfaces of villous enterocytes and within cytoplasmic vacuoles in two of four piglets inoculated with WAM2335 and from one of three piglets inoculated with WAM2317. They were within cytoplasmic vacuoles in two of four piglets inoculated with WAM2335 and from one of three piglets inoculated with WAM2317.
Numerous bacteria are shown within a large cytoplasmic vacuole in an enterocyte. Bar = 1 μm.

Vacuoles in these cells. Bacteria on the surfaces of enterocytes were either loosely or intimately adhered to microvilli. Occasionally, intimately adhered bacteria were seen in invaginations in the apical surfaces of enterocytes with disrupted microvilli. Individual bacteria were present in small cytoplasmic vacuoles (data not shown), whereas multiple bacteria were often present in large cytoplasmic vacuoles (Fig. 4). Bacteria also were seen within cytoplasmic vacuoles of M cells (data not shown).

In this study we provide the first report of the construction of an Hly 

isogenic mutant of a porcine ETEC strain. The hlyA gene in this strain was inactivated by double homologous recombination following introduction of an HlyC

ΔhlyA fragment on a conjugatable suicide plasmid. The occurrence of a deletion event in hlyC in conjunction with Km

cassette insertion into this region was confirmed by Southern blot analysis.

We hypothesized that inactivation of hlyA would decrease the ability of ETEC to cause secondary septicemia in swine. However, inactivation of hlyA did not reduce the incidence of diarrhea or septicemia in neonatal gnotobiotic piglets inoculated with isogenic Hly

and Hly

strains. Our results confirm and extend those of Smith and Linggood, who, by using an ETEC strain cured of its Hly plasmid, found that hemolysin played no role in diarrhea (33). Either hemolysin plays no role in septicemia in this model or other virulence factors obscured its effects. Animal-to-animal variation also may have influenced the outcome (21) and may explain why only one of three piglets inoculated with the Hly

parent strain developed septicemia despite the fact that all three had bacterial colonization of the lungs.

The finding of ETEC within cytoplasmic vacuoles of columnar enterocytes may reflect the predisposition of neonatal gnotobiotic piglet intestine to phagocytose bacteria and/or an invasive capacity of the serotype used for inoculation (16, 19, 25, 30, 35). ETEC is not considered to be invasive in the classical sense (26). However, the results of studies of natural and experimental infections in piglets less than 1 week old suggested that ETEC serotype O8:K87 (previously termed G7), in particular, is invasive (16, 19, 30, 35). Ultrastructural examination of the intestine was not done in these previous studies with E. coli O8:K87, and to our knowledge, E. coli bacteria within cytoplasmic vacuoles of enterocytes had not been seen in piglets inoculated with other ETEC serotypes. However, they have been seen in gnotobiotic piglets inoculated with enteropathogenic E. coli, enterohemorrhagic E. coli, and septicemia-inducing E. coli (21, 25, 37, 41). Interestingly, more recent studies have shown that a classical human ETEC strain (H10407) invades human intestinal cell lines and that the capacity for invasion is located on two separate, chromosomal loci, designated tia and tib (toxigenic invasion loci A and B [10]). Based on our results and those from other studies, we hypothesize that intestinal invasion plays a role in the pathogenesis of some ETEC infections.

A criterion important in order for an extraintestinal infection to occur is the ability of the strain to survive and to persist in the bloodstream and extraintestinal tissues (8). In the present study, both the nonhemolytic mutant and hemolytic parent strain survived and caused bacterial septicemia in the process. Hypothetically, hemolysin might enhance the ability of E. coli to survive in the bloodstream by mediating resistance to complement and phagocytes and by serving as an alternative mechanism for the acquisition of iron in the absence of aerobactin (31, 43). Further studies with the enterically infected gnotobiotic piglet model could address the effects of hemolysin on extraintestinal infection. These include competition experiments with Hly

and Hly

organisms, studies of the effects of lower inoculum levels, studies addressing the time of onset and level of bacteremia, and studies of the effects of indigenous enteric bacteria (e.g., Lactobacillus spp. and Streptococcus faecium).

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