

Antibodies against Affinity-Purified, Surface-Exposed Outer Membrane Proteins of *Edwardsiella ictaluri* Block Invasion into Fathead Minnow Epithelial Cells

RAMONA T. SKIRPSTUNAS* AND THOMAS J. BALDWIN

Department of Animal, Dairy, and Veterinary Science,
Utah State University,
Logan, Utah 84322, USA

Abstract.—Surface-exposed outer membrane proteins (OMPs) of *Edwardsiella ictaluri* were isolated by selective solubilization of inner membrane proteins from total membrane preparations. Purification of biotin-labeled, insoluble, surface-exposed proteins using streptavidin columns was performed, and single-dimension sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) showed four major OMPs, with apparent molecular weights of 22, 31, 59, and 72 kilodaltons (kDa). Purified surface-exposed proteins corresponded to proteins isolated from total outer membrane preparations resolved by SDS–PAGE, showing that surface-exposed proteins are within the outer membrane fraction and can be successfully isolated using affinity purification. Polyclonal antiserum against these surface-exposed OMPs was produced in New Zealand white rabbits, and protein recognition was determined using in-gel Western analysis. Rabbit antisera recognized three of the four protein bands (22, 31, and 59 kDa). The produced antisera blocked invasion of cells from fathead minnow *Pimephales promelas* by virulent *E. ictaluri*, showing that at least one of these proteins is involved in initial bacterial–host cell interactions.

Edwardsiella ictaluri is a gram-negative bacterium in the family Enterobacteriaceae that causes enteric septicemia of catfish (ESC; Hawke 1979; Hawke et al. 1981), the most economically important bacterial disease among commercially produced channel catfish *Ictalurus punctatus*. Efforts to control ESC using various vaccination preparations and the few antibiotics approved for food fish have proven unsuccessful (Plumb et al. 1986; Saeed and Plumb 1986; Thune et al. 1993, 1997a, 1997b). The lack of control is due in part to an incomplete understanding of the pathogenesis of ESC, compounded by ineffective vaccine formulations and administration strategies (and Saeed and Plumb 1986). Identification of the bacterial molecules involved in the initial invasion of host cells will clarify the pathogenesis of ESC and identify

potential target antigens for ongoing vaccination efforts.

In an effort to identify the components of *E. ictaluri* involved in host cell adherence and internalization, the invasion strategies of other Enterobacteriaceae members were considered. Within this family, most of the molecules mediating bacterial adhesion, and in some cases internalization, are surface-exposed proteins that reside (permanently or temporarily) within the bacterial outer membrane (Miller et al. 1988; Finlay and Falkow 1989; Pulkkinen and Miller 1991). These proteins serve as membrane-bound ligands and/or secreted products that allow for the exploitation of normal host cell mechanisms, resulting in bacterial uptake (Isberg et al. 1987; Finlay and Falkow 1989; Finlay et al. 1991). The functions of the outer membrane proteins of *E. ictaluri* are largely unknown, although based on data gathered from other members of this family of pathogens, surface-exposed proteins are suspected of being major contributors to host cell invasion.

Using the American Type Culture Collection (Rockville, Maryland) reference isolate *E. ictaluri* 33202, Newton et al. (1990) identified one major outer membrane protein (molecular weight, 35 kilodaltons [kDa]) and nine minor proteins (71, 51, 46, 43.5, 38.5, 37.5, 31.5, 28.5, and 19.5 kDa). Two outer membrane proteins were identified using immune catfish serum in each of two separate studies; these proteins had molecular weights of 39 and 37 kDa (Baldwin et al. 1997) and 34 and 60 kDa (Plumb and Klesius 1988) in the two studies, respectively. Klesius and Horst (1991) used monoclonal antibodies to identify 36- and 60-kDa antigens from *E. ictaluri* and showed that these proteins were localized on the bacterial surface. Although antigenicity was evaluated in these studies, the involvement of these proteins in initial host cell interactions was not addressed, in part due to the lack of a functional in vitro cell model system. Identification of several cultured cell lines susceptible to *E. ictaluri* invasion has been described

* Corresponding author: rskirps@cc.usu.edu

(Skirpstunas and Baldwin 2002). Susceptible cultured cells provide the necessary model system to study initial bacterial–host cell interactions.

In this study, surface-exposed proteins of *E. ictaluri* were isolated and polyclonal antisera produced against the purified proteins. Antiserum was subsequently used as a primary blocking reagent to determine whether the isolated proteins were involved in initial bacterial–host cell interactions. Invasion-blocking assays were performed with one of the recently defined *in vitro* systems, utilizing cells from fathead minnow *Pimephales promelas* (Skirpstunas and Baldwin 2002). This study provides the first evidence of invasion blocking of *E. ictaluri* into the epithelial cells of cultured fish using antibodies produced against surface-exposed bacterial outer membrane proteins.

Methods

Bacterial isolates and media.—By way of preparation, *E. ictaluri* (laboratory isolate LA 89-9) were serially passed through fingerling channel catfish by gastric intubation and subsequent recovery from kidney cultures (three times). The isolate was stored in brain–heart infusion broth (BHIB; Gibco BRL Life Technologies, Inc., Gaithersburg, Maryland) containing 20% glycerol at -20°C . Prior to use, the bacteria were amplified in BHIB at 26°C with gentle agitation for 24 hours.

Biotinylation of surface-exposed proteins of *E. ictaluri*.—After being amplified in BHIB, *E. ictaluri* cells were pelleted by centrifugation at $2,000 \times$ gravity [g] for 5 min at 4°C and then washed in ice-cold calcium- and magnesium-free phosphate-buffered saline (CMF: 0.14 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO_4 , 0.001 M KH_2PO_4 ; pH 7.4) three times. Bacteria were resuspended in CMF to a concentration of approximately $1 \times 10^{10}/\text{mL}$, as determined by prior dilution plate counts. Bacteria were exposed to sulfo-*N*-hydroxysuccinimide-biotin (Sigma, St. Louis, Missouri) at a concentration of 0.5 mM in CMF for 60 min at 4°C with gentle agitation. Nonbound biotin was blocked by adding an equal volume of 10-mM glycine in CMF followed by incubation for 20 min at room temperature. Biotinylated bacteria were washed four times as described above and either used immediately or stored in phosphate-buffered saline (PBS) at -20°C .

Outer membrane protein preparation.—The procedures used to isolate outer membrane proteins of *E. ictaluri* were similar to those reported by Newton et al. (1990) and Baldwin et al. (1997); they employed the selective solubilization of inner mem-

brane components with sodium lauroyl sarcosine (SLS; Sigma), leaving the insoluble, outer membrane components. Briefly, following biotinylation, bacteria were collected by centrifugation at $2,000 \times g$ for 20 min at 4°C . The pelleted bacteria were resuspended in 10 mL of 10-mM HEPES buffer (*N*-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, pH 7.4), placed in an ice bath, and sonicated (Model 300 sonicator; Fisher Scientific, Pittsburgh, Pennsylvania) four times for 30 s each. Intact cells and large debris were removed by centrifugation at $5,000 \times g$ for 20 min at 4°C . Fragmented cell membranes were harvested from the supernatant by centrifugation at $100,000 \times g$ for 1 h at 4°C . The pellet was resuspended in 1 mL 10-mM HEPES buffer (pH 7.4) and inner membrane proteins solubilized by the addition of 10 mL of 1.5% SLS in 10-mM HEPES buffer for 30 min at room temperature. The detergent-insoluble (outer membrane) fraction was harvested by centrifugation at $100,000 \times g$ for 1 h at 4°C and resuspended in distilled water. The final protein solution was adjusted to a concentration of 2.0 mg/mL, as determined by the bicinchoninic acid (BCA) microprotein assay (Pierce, Rockford, Illinois) using distilled water. Outer membrane proteins were stored in distilled water at -20°C .

Affinity purification of biotinylated outer membrane protein fraction.—The OMP fraction of *E. ictaluri* (2 mg total protein/mL) was enriched for labeled, surface-exposed proteins using an affinity column containing immobilized streptavidin (Pierce) following the manufacturer's instructions. Briefly, the column was equilibrated with five column volumes of PBS. The sample solution was added to the column and allowed to incubate for 30 min to 1 h. The column was washed with 10 column volumes of binding buffer (50 mM ammonium carbonate and 500 mM NaCl; pH 11). After washing, bound protein was eluted using 5–10 column volumes of elution buffer (50 mM dithiothreitol). Dithiothreitol breaks the disulfide bond between the long-chain spacer arm connecting the streptavidin–biotin complex and the protein itself. The eluted protein fraction was collected, dialyzed overnight against purified distilled water, and adjusted to a concentration of approximately 200 $\mu\text{g}/\text{mL}$ using the BCA protein assay kit (Pierce). Aliquots were stored in distilled water at -20°C .

Rabbit immunizations.—Polyclonal antisera were produced against 25–50 μg of purified OMPs. Purified protein preparations were emulsified with Ribi Adjuvant System–monophosphoryl lipid A (Ribi ImmunoChem Research, Inc., Hamilton,

Montana) as recommended to a final volume of 1.0 mL at each vaccination time. One milliliter (each) was injected into two New Zealand white rabbits, 0.5 mL subcutaneously, 0.3 mL intramuscularly, and 0.1 mL intradermally (at two sites). Three boosts of 50 µg of eluted protein in RAS-MPL followed every 28 d, for a total of 4 immunizations. Serum was obtained 14 d after each boost by auricular venipuncture.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).—SDS-PAGE was performed using the NuPage system (Invitrogen, San Francisco, California) as directed by the manufacturer. Commercially available protein standards (See Blue, Invitrogen) were run alongside bacterial samples. Gels were silver stained using the Silver Xpress staining kit (Invitrogen) to identify outer membrane protein profiles.

Western immunoblot.—Immunoblot screening was performed to verify the specificity of the rabbit antisera produced against affinity-purified OMPs. Total outer membrane protein preparations were separated using standard SDS-PAGE, and an in-gel Western blot was performed using the Unblot Western analysis kit (Pierce). After a 30-min exposure to a 50:50 solution of isopropanol and water, gel strips were probed with a 1:25 dilution of sera from immunized rabbits (primary antibody) for 1 h at room temperature with mild agitation. The gel strips were washed three times for 10 min with a tris-buffered saline -0.5% Tween solution, after which secondary antibody (goat antirabbit immunoglobulin G labeled with horseradish peroxidase) was applied for 1 h at room temperature. The gel strips were washed as above and exposed to substrate solution enhanced with luminol (Pierce) for 20 s. The gel strips were then rinsed with distilled water, placed between cellophane sheets, and exposed to film for band visualization. Preimmune rabbit antiserum was used as a negative control, as were sample lanes exposed to primary or secondary antibody only.

Lipopolysaccharide (LPS) adsorption of antiserum.—Antibodies against LPS were removed from test antisera by mixing antisera with an equal volume of purified *E. ictaluri* (LA 89-9) LPS solubilized at 1 mg/mL in CMF and incubating the mixture at room temperature for 1 h and then at 4°C for 6 h. Antibody-LPS complexes were pelleted by centrifugation at $10,000 \times g$ for 5 min at room temperature, and the pellet was discarded (Baldwin et al. 1997). The LPS was purified using enzyme digestion and hot aqueous phenol extraction as previously described (Westphal and Jann

1965). Before use, all antisera were heat inactivated at 56°C for 30 min and diluted to no more than 1:25.

Invasion assays.—The fathead minnow (FHM) cell line was described previously as a functional in vitro system for the study of *E. ictaluri* pathogenesis (Skirpstunas and Baldwin 2002). Briefly, bacteria were incubated with epithelial cells, followed by selective killing of extracellular bacteria by Gentamicin (Gibco BRL). Internalized bacteria were subsequently released by lysis of the epithelial cells with a detergent solution (Rubens et al. 1992; Ellinghorst 1994). Invasion assays were performed in 24-well plates (Falcon Primaria, Franklin Lakes, New Jersey) by incubating $6-8 \times 10^6$ colony-forming units (CFUs) of *E. ictaluri* or *Escherichia coli* DH5 α (noninvasive control) organisms with confluent FHM cells (approximately 4.0×10^5 cells/well) for 2 h (the invasion period) at 26°C in normal atmosphere. The medium was removed and the cells washed twice with CMF. Medium containing 100 µg/mL Gentamicin was applied and cells incubated for 4 h at 26°C (the extracellular killing period). Cells were washed four times with CMF and 100 µL of the final wash plated onto brain-heart infusion agar or *E. ictaluri*-selective media to ensure effective killing or removal of extracellular bacteria. Trypan blue was added to the wells and viable cell counts performed prior to cell lysis. The fluid overlying the cells was removed and cells were lysed with 200 µL of 1% Triton X-100 (Amersham, Arlington Heights, Illinois) in CMF. After 10 min, samples of the cell lysate were triturated using a transfer pipette, serially diluted, plated on *E. ictaluri*-selective media (Shotts and Waltman 1990) or MacConkey's agar (*E. coli*), and incubated at 26°C for intracellular CFU determination. Cells that were incubated with the bacteria but that did not receive Gentamicin ensured bacterial viability throughout the experiment. Cells that were taken through all assay steps except having bacteria added ensured that the cultured cells were free of contaminating intracellular bacteria and not damaged by washing or the antibiotic treatments.

Invasion-blocking assays were performed by incubating $3-4 \times 10^6$ cells of *E. ictaluri* and *E. coli* with a 1:4 dilution of anti-*E. ictaluri*-purified OMP antisera for 40 min at room temperature with mild agitation. Test groups included *E. ictaluri* and *E. coli* that were preincubated with antiserum, incubated with preimmune antiserum from the same rabbit (control for nonspecific inhibition), or un-

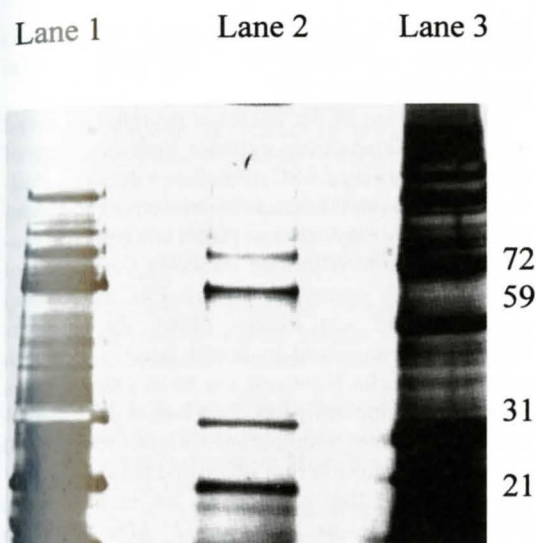


FIGURE 1.—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins (OMPs) of *Edwardsiella ictaluri*. Lane 1 shows the results for the total OMP preparations isolated by means of the sodium lauroyl sarcosine solubilization procedure, lane 2 the results for biotin-labeled, surface-exposed, affinity-purified OMPs, and lane 3 the molecular weight standard.

treated (control for active invasion). Assays were otherwise run as described above.

Statistics.—All invasion and blocking assays were performed in triplicate and the experiments repeated independently three times. All data were expressed as the mean and standard error or standard deviation. Data in blocking assays were compared with untreated controls using Student's *t*-test with a significance level of 0.05.

Results

Biotinylation of surface-exposed outer membrane proteins of *E. ictaluri* with subsequent affinity purification yielded four proteins (with molecular weights of 21, 31, 59, and 72 kDa) coinciding with the protein bands separated from the total outer membrane protein preparations (Figure 1). Three of these (21, 31, and 59 kDa) were recognized by serum from both rabbits; as the quantity of antibody produced was greater in one rabbit than in the other, however, its serum was employed in the blocking experiments. These data indicate that surface proteins of *E. ictaluri* can be successfully labeled and isolated and that three of the four proteins are immunogenic when emulsified with Ribi adjuvant and administered to New Zealand white rabbits.

Incubation of *E. ictaluri* with antiserum directed

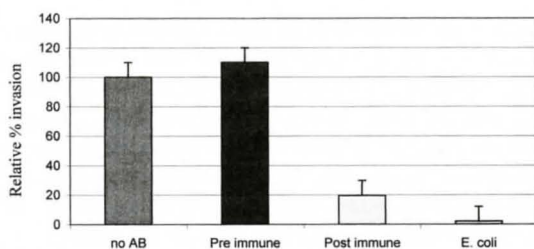


FIGURE 2.—Relative percent invasion of fathead minnow cells by virulent *E. ictaluri*. Untreated bacteria (no AB) were considered to have a relative invasion of 100%. Shown are the means and standard deviations of three independent invasion-blocking trials (incubation of *E. ictaluri* and *Escherichia coli* with rabbit anti-*E. ictaluri*-purified outer-membrane protein antiserum [postimmune and *E. coli*, respectively] and incubation of *E. ictaluri* with preimmune antiserum from the same rabbit [preimmune]), each performed in triplicate.

against surface-exposed outer membrane proteins reduced the invasion of virulent bacteria by 80.4% (Figure 2) compared with *E. coli* untreated with antiserum (the negative invasion control). The invasion-blocking effect was negated by antiserum dilutions of 1:8 and above, suggesting that invasion blocking depends on the specific antibody concentration (Figure 3).

Discussion

Evaluation of the initial interactions between *E. ictaluri* and susceptible host cells has until now been hampered by the lack of a functional in vitro assay system, leaving the pathogenesis of *E. ictaluri* at the molecular level virtually unknown. Identification of cultured cell lines that are susceptible to *E. ictaluri* invasion provided the needed model systems (Skirpstunas and Baldwin 2002).

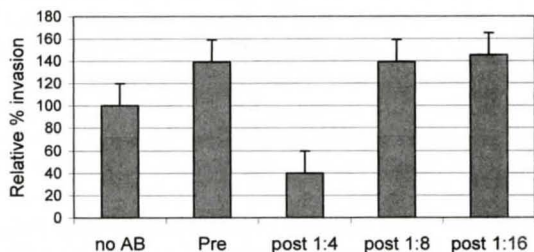


FIGURE 3.—Relative percent invasion of fathead minnow cells by *E. ictaluri*. Untreated bacteria (no AB) were considered to have a relative invasion of 100%. Bacteria pretreated with preimmune antiserum (pre) and three dilutions of rabbit anti-*E. ictaluri*-purified outer-membrane protein antiserum were compared. Shown are the means and standard errors of two independent invasion-blocking trials, each performed in quadruplicate.

Although the number of outer membrane proteins evaluated for potential interaction with host cells can be limited by selective solubilization of inner membrane components, the labeling and affinity purification of surface-exposed proteins provide a means of evaluating a small number of suspect molecules in detail. Isolation and identification of such proteins was the first goal of this study.

The second goal was to produce an antiserum against these purified proteins for use as blocking reagents and to evaluate the role of these proteins in host cell invasion. The proteins were isolated using a biotin labeling technique and affinity purification. Biotin complexes are small and react under mild conditions, making this procedure ideal for labeling the surface proteins of intact cells and allowing native protein interactions to occur (Cole et al. 1987; Bayne and Hull 1988; Bhakdi et al. 1989).

If the identified proteins function as ligands for receptors or are necessary for the adherence leading to bacterial internalization, an antibody against those proteins can prevent binding to specific cell receptors. The quantity and specificity of the antibodies produced against different protein epitopes may interfere with this process, as the immunodominant regions may not be those involved in receptor binding. However, production of a polyclonal antibody maximizes the variety of antibodies produced so as to better cover the epitopes exposed on these proteins. Another complicating factor is that antibody-recognizing specific proteins may not directly abate internalization if the proteins are involved in adherence only. Adherence and internalization are coupled in the assays used in this study and are considered a cause-and-effect relationship rather than being examined independently. Additionally, more than one adhesion-internalization system may exist, and different cell systems and types may use different mechanisms of invasion. In any event, the blocking of internalization by specific antibodies that recognize the identified surface-exposed proteins pinpointed specific molecules on which to focus, namely, the ones most likely to be involved in initial bacterial-host cell interactions.

In this study, the cells of rabbits immunized with purified *E. ictaluri* proteins recognized three of the four surface-exposed OMPs. Recognition of only three proteins may be due either to an inherently nonimmunogenic nature of the 72-kDa protein or the failure of the antisera produced from the purified protein to recognize the reduced denatured form as separated by SDS-PAGE. Blocking assays show that one, two, or all three of these proteins

contribute to the internalization of *E. ictaluri* into FHM cells, and the effect is abolished by serial twofold dilutions at 1:8.

Identification of the bacterial proteins involved in host cell interactions expands understanding of the pathogenesis of ESC and allows detailed studies of the proteins themselves. Moreover, the identified proteins may serve as target antigens for vaccine production. Although antibody levels do not correlate with protection against ESC in channel catfish (Saeed and Plumb 1986), the proteins known to be involved in initial interactions with susceptible cells have not yet been employed as primary vaccine antigens. Previous work characterizing the immunodominant antigens recognized by catfish immune serum identified bands with estimated molecular weights of 34 and 60 kDa (Plumb and Klesius 1988) and 37 kDa (Baldwin et al. 1997). Whether the proteins identified in this study coincide with those recognized by catfish serum in other reports remains to be determined.

Acknowledgments

Appreciation is extended to Ron Silflow for maintenance of the cultured cell lines and bacterial isolates used in this study. This work was supported by the U.S. Department of Agriculture (NRI 00-35204-9211).

References

- Baldwin, T. J., L. A. Collins, and J. C. Newton. 1997. Antigens of *Edwardsiella ictaluri* recognized by serum antibodies from naturally infected channel catfish. *Fish and Shellfish Immunology* 7:261-271.
- Bayne, C. J., and C. J. Hull. 1988. The host-parasite interface in Molluscan schistosomiasis: biotin as a probe for sporocyst and hemocyte surface peptides. *Veterinary Parasitology* 29:131-142.
- Bhakdi, S., M. Roth, and F. Hugo. 1989. Biotinylation: a simple method for labeling complement component C8 with preservation of functional activity. *Journal of Immunological Methods* 121:61-66.
- Cole, S. R., L. K. Ashman, and P. L. Ey. 1987. Biotinylation: an alternative to radioiodination for the identification of cell surface antigens in immunoprecipitates. *Molecular Immunology* 24:699-705.
- Ellinghorst, E. A. 1994. Measurement of invasion by Gentamicin resistance. *Methods in Enzymology* 236:405-420.
- Finlay, B. B., and S. Falkow. 1989. Common themes in microbial pathogenicity. *Microbiological Reviews* 53:210-230.
- Finlay, B. B., S. Ruschkowski, and S. Dedhar. 1991. Cytoskeletal rearrangements accompanying *Salmonella* entry into epithelial cells. *Journal of Cell Science* 99:283-296.
- Hawke, J. P. 1979. A bacterium associated with disease

- of pond cultured channel catfish (*Ictalurus punctatus*). Journal of the Fisheries Research Board of Canada 36:1508-1512.
- Hawke, J. P., A. C. McWhorter, and A. G. Steigerwalt. 1981. *Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish. International Journal of Systemic Bacteriology 31:396-400.
- Isberg, R. R., D. L. Voorhis, and S. Falkow. 1987. Identification of invasins: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell 50:769-778.
- Klesius, P. H., and M. Horst. 1991. Characterization of a major outer-membrane antigen of *Edwardsiella ictaluri*. Journal of Aquatic Animal Health 3:181-187.
- Miller, V. L., B. B. Finlay, and S. Falkow. 1988. Factors essential for penetration of mammalian cells by *Yersinia*. Current Topics in Microbiology and Immunology 138:15-39.
- Newton, J. C., W. T. Blevins, G. R. Wilt, and L. G. Wolfe. 1990. Outer membrane protein profile of *Edwardsiella ictaluri* from fish. American Journal of Veterinary Research 51:211-215.
- Plumb, J. A., and P. H. Klesius. 1988. An assessment of the antigenic homogeneity of *Edwardsiella ictaluri* using monoclonal antibody. Journal of Fish Diseases 11:499-509.
- Plumb, J. A., M. L. Wise, and W. A. Rogers. 1986. Modulatory effects of temperatures on antibody response and specific resistance to challenge of channel catfish (*Ictalurus punctatus*) immunized against *Edwardsiella ictaluri*. Veterinary Immunology and Immunopathology 12:297-304.
- Pulkkinen, W. S., and S. I. Miller. 1991. A *Salmonella typhimurium* virulence protein is similar to a *Yersinia enterocolitica* invasion protein and a bacteriophage Lambda outer membrane protein. Journal of Bacteriology 173:86-93.
- Rubens, C. E., S. Smith, M. Hulse, E. Chi, and G. van Belle. 1992. Respiratory epithelial cell invasion by group B streptococci. Infection and Immunity 60:5157-5163.
- Saeed, M. O., and J. A. Plumb. 1986. Immune response of channel catfish to lipopolysaccharide and whole cell *Edwardsiella ictaluri* vaccines. Diseases of Aquatic Organisms 2:12-26.
- Shotts, E. B., and W. D. Waltman. 1990. A medium for the selective isolation of *Edwardsiella ictaluri*. Journal of Wildlife Diseases 26:214-218.
- Skirpstunas, R. T., and T. J. Baldwin. 2002. Invasion of IEC-6, Henle 407, fat head minnow and channel catfish enteric epithelial cells by *Edwardsiella ictaluri*. Diseases of Aquatic Organisms 51:161-167.
- Thune, R. L., L. A. Collins, and M. P. Pena. 1997a. A comparison of immersion, immersion/oral combination, and injection methods for the vaccination of channel catfish *Ictalurus punctatus* against *Edwardsiella ictaluri*. Journal of the World Aquaculture Society 28:193-201.
- Thune, R. L., J. P. Hawke, D. H. Fernandez, M. L. Lawrence, and M. M. Moore. 1997b. Immunization with bacterial antigens: edwardsiellosis. Developments in Biological Standardization 90:125-134.
- Thune, R. L., J. P. Hawke, and M. C. Johnson. 1993. Studies on vaccination of channel catfish, *Ictalurus punctatus*, against *Edwardsiella ictaluri*. Journal of Applied Aquaculture 3:11-23.
- Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharide extraction with phenol-water and further applications of the procedure. Pages 83-96 in R. L. Whistler, editor. Methods of carbohydrate chemistry. Academic Press, New York.