CONSTRUCTION AND CHARACTERIZATION OF A PLASMID DNA LIBRARY FROM EDWARDSIELLA ICTALURI

CONTESSA MORGAN-WOOD

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Date April 21, 1997

Construction and Characterization of a Plasmid DNA Library from *Edwardsiella ictaluri*

A Thesis

Presented for the

Master of Science

Degree

Austin Peay State University

Contessa Morgan-Wood

April, 1997

ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Dailey, for his patience and guidance over the last two and a half years. I would like to thank my other committee members, Dr. Taylor and Dr. Lebkuecher for their advice and assistance on this project. I would also like to thank Mrs. Lyle for her help and creativity with scanning picture images in the computer. I thank my husband, Charlie, for his understanding, patience and humor during those times when I felt like giving up. I wish to thank my brother, Chad, and his wife Carla, for their support. Finally, I would like to express my thanks to my parents, Randall and Nancy Morgan, for their continual support and guidance.

Abstract

Enteric septicemia is the most serious bacterial disease in commercially raised channel catfish, *Ictalurus punctatus*. The gram-negative facultative anaerobic bacterium responsible for this disease is *Edwardsiella ictaluri*. Since *Ed. ictaluri* was first discovered in 1976, much of the research has demonstrated the commonality of various isolates of *Ed. ictaluri* based on biochemical characteristics and plasmid DNA profiles. There are no studies correlating plasmid DNA of *Ed. ictaluri* with virulence of the bacterium. To understand the biology of the plasmids in *Ed. ictaluri*, a DNA fragment library representative of the plasmid DNA was constructed. Overlapping fragments of the pCL 1 plasmid were cloned into the plasmid vector pTZ18U. Restriction endonuclease digestion followed by Southern analysis was used to confirm the identity of four unique classes of recombinant clones. Southern analysis of chromosomal DNA isolated from *Ed. ictaluri* with the pCL 1 plasmid and recombinant DNA clones did not reveal any sequence homology between chromosomal and pCL 1 plasmid DNA. Northern analysis of *Ed. ictaluri* failed to demonstrate expression of genes encoded by the pCL 1 plasmid under optimal growth conditions. The recombinant plasmid clones were then used in plasmid incompatibility experiments to cure *Ed. ictaluri* of plasmid DNA. Transformation of competent *Ed. ictaluri* cells with recombinant clones, pA10 and pB1, cured *Ed. ictaluri* of plasmid DNA. Agarose gel electrophoresis of DNA isolated from successful transformants of *Ed. ictaluri* revealed a 9.4 kb band. The size of the 9.4 kb band and hybridization with a pTZ18U gene probe indicates that recombination between the

recombinant DNA molecule and pCL 1 has occurred. Neither pCL 1 nor pCL 2 were observed in the transformed cells indicating these plasmids were successfully cured from *Ed. ictaluri*.

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Introduction

Edwardsiella ictaluri is a Gram-negative, facultative anaerobic bacterium that belongs to the family Enterobacteriaceae (9). This bacterium is rod-shaped, 0.5 by 1.25 μm, oxidase-negative, and exhibits peritrichous flagellation (9, 36). The optimal *in vitro* growth temperature of this bacterium is between 25 to 30 °C. At 30 °C, growth of this organism on blood agar is slow and usually requires 48 h to form typical colonies of 2 mm in diameter (9).

Edwardsiella ictaluri is the bacterial pathogen responsible for enteric septicemia of channel catfish (ESC), *Ictalurus punctatus* (16). Enteric septicemia of channel catfish was first discovered in 1976 by Hawke et. al. at the Southeastern Cooperative Fish Disease Laboratory, Auburn University (8). Enteric septicemia is considered the most serious bacterial disease in commercially raised channel catfish (20) because it can progress rapidly in apparently healthy, fast-growing catfish and can result in extensive mortality (15). Enteric septicemia is predominantly a disease of fingerlings although all ages of catfish can become infected (4, 22). Furthermore, only older catfish have been found to be capable of developing immunity (4).

Outbreaks of enteric septicemia have been determined to occur within the optimum *in vitro* growth temperature of the bacterium, which usually occurs in the early summer and autumn months (4, 15, 16, 36). Within this temperature range, the incidence of disease-associated mortality is high. When water temperatures move above or below this temperature range, the bacteria enter a dormant phase in the macrophage of the

catfish or in bottom sediments. When *Ed. ictaluri* is dormant in pond mud, the bacterium can survive for at least 90 days. This allows the bacterium to survive the extremes of the winter and summer months when the temperature is not optimal for *Ed. ictaluri* growth. Survival in bottom sediments enables this bacterium to infect healthy fish once the water temperatures reenter the optimal range (4). Enteric septicemia of channel catfish can develop in two forms: 1) an acute septicemia with rapid mortality and 2) a chronic granulomatous meningoencephalitis, often referred to as "hole in the head" disease (30). The route of infection for the acute stage of disease is through the gastrointestinal tract from which it rapidly disseminates throughout the body. Macrophages have been implicated in the dissemination of *Ed. ictaluri* throughout the fish. Clinical signs of enteric septicemia are small red and white spots that cover the entire body, a pale anemic appearance due to the destruction of red blood cells, and abnormal swimming. In the chronic form of disease, route of infection is through the nares from which the bacteria invades the brain and skull (19, 30). The establishment of chronic infection in the brain and subsequent production of chondroitinase may result in the characteristic "hole-in-the-head" lesion (30). The hole-in-the-head lesion presents initially as a raised red spot on the head of the catfish followed by massive tissue destruction (4, 22).

Studies characterizing the plasmid content of *Ed. ictaluri* isolated from channel catfish, have consistently shown only two plasmids (16, 25, 32). These two plasmids have been determined to be about 5.7 kb and 4.9 kb in length and have been designated pCL 1 and pCL 2 respectively (16, 25, 32). When analyzed by restriction endonuclease digestion the plasmids have been found to be distinct from one another (16). When analyzed by

cross hybridization, pCL 2 probes did not cross hybridize with pCL 1 indicating that the plasmids do not share sequence homology (15). Also plasmid DNA labeled as probes and hybridized to *Ed. ictaluri* chromosomal DNA have failed to demonstrate any sequence homology (15, 32). Because both plasmids are maintained in all isolates of *Ed. ictaluri* and no sequence similarity has been demonstrated between the plasmid classes, both plasmids are viewed as distinct and very important to *Ed. ictaluri.*

Plasmid DNA of many pathogenic Gram negative bacteria have been shown to encode properties essential to the virulence of the bacterium. Such properties may include adherence/invasion factors, intrahost bacterial survival factors, and disease provoking factors (11). In *Yersinia* species, plasmid DNA enhances virulence and promotes intrahost multiplication and survival by encoding resistance to complement-mediated killing and phagocytosis as well as encoding for invasion and dissemination within the host (11). For example, a 41-megadalton plasmid in *Y. enterocolitica* is responsible for encoding tissue invasiveness in human enteric disease (5). In addition the *yad* A gene on a 70 kb virulence plasmid of *Y. pseudotuberculosis* and *Y. enterocoliticia* has been shown to have multiple virulence functions such as attachment and invasion of cultured mammalian cells, serum resistance and autoagglutination (38). In *Salmonella typhimurium,* a 60 Mdal plasmid was found to be associated with the adherence and invasion of virulent phenotypes. When the 60 Mdal plasmid was present, the *S. typhimurium* bacterium could adhere and invade HeLa cells. Once the bacteria were cured of this 60 Mdal plasmid, the bacteria demonstrated decreased adherence to HeLa cells and when injected into mice they were approximately 1,000 to 10,000 fold less virulent (10). Similar studies comparing

strains of *Shigella flexneri* and *Escherichia coli* found a 140 Mdal plasmid that was necessary for cell penetration (6, 27). Finally, a 47 Mdal plasmid was characterized in *Vibrio anguillarum* to encode for an iron uptake system which enables the bacterium to grow under iron limited conditions (23). These data clearly document the importance of plasmid DNA to bacterial survival and pathogenesis.

Even though plasmid DNA has been demonstrated in *Ed. ictaluri* since 1987 (16), there have been no studies linking virulence of *Ed. ictaluri* with the presence of plasmid DNA. However, *Edwardsiella spp.*, including *Ed. ictaluri*, have been shown to be resistant to the bactericidal effects of 20 % serum (24). In many Gram negative bacteria, resistance to serum has been shown to be encoded by plasmid DNA and to correlate with the ability of the bacteria to invade and survive in the bloodstream (33). As stated previously the *yad* A gene in *Y. .pseudotuberculosis* is encoded by a plasmid and is linked to serum resistance (38). Serum resistance may assist the bacterium by complementing phagocytic components of macrophages, and by altering the ability of serum binding proteins of macrophages such as albumin, fibrogen, and IgG to destroy the bacteria (18). A study by Shotts et. al. on the pathogenesis of *Ed. ictaluri* in catfish, found macrophages containing the bacteria in every tissue examined (30). Given that plasmids of Gram negative bacteria encode for serum resistance and that *Ed. ictaluri* is resistant to serum, it is possible that *Ed. ictaluri* plasmid DNA is responsible for serum resistance and may explain the bacterium's ability to survive within the macrophages of catfish.

Plasmid curing is an effective method of documenting the importance of plasmid

DNA to the virulence of the bacterium (1, 34, 35). Some of the common methods used to cure a bacterium of plasmid DNA include plasmid incompatibility (34, 35), the removal of selectable markers (1), and growth of the bacterium in chemical mutagens (12, 29). Plasmid incompatibility refers to the inability of two different plasmids containing the same origin of replication to coexist in the same cell (21). Valla et. al. developed a plasmid curing method which involved the use of plasmid incompatibility to construct plasmid-free derivatives of *Vibrio anguillarum* for virulence studies (34). Other studies have demonstrated that in the absence of antibiotic selection, virulence plasmids of many *Salmonella* spp. can be cured from the cell (2, 13, 14). Furthermore, chemical mutagens, such as ethidium bromide, were capable of removing a 95 kb plasmid from virulent strains of *E. coli* which resulted in decreased virulence of this bacterium in chickens (28). One study on *Ed. ictaluri* has addressed the curing of plasmid DNA (16). The study briefly discussed unsuccessful attempts to cure plasmid DNA from *Ed. ictaluri* by growth at elevated temperatures (16).

Research on *Ed. ictaluri* has demonstrated the commonality of plasmid DNA among various isolates, the absence of any unique biochemical characteristics of these isolates and the restricted pathogenicity of the bacterium to channel catfish (15, 16, 19, 20, 25, 30, 32, 36). There have been no studies correlating plasmid DNA to any virulence properties of the bacterium. As stated earlier, *Ed. ictaluri* demonstrates the ability to resist the bactericidal effects of serum which in many Gram negative bacteria is a virulence factor encoded by plasmid DNA (24, 38). Therefore, my study begins to characterize *Ed. ictaluri* plasmid DNA by constructing a DNA fragment library representative of the

plasmid DNA of *Ed. ictaluri*. Segments of *Ed. ictaluri* plasmid DNA were cloned into the plasmid vector pTZ18U and the recombinant molecules transformed into *E. coli* RR1ΔM15. Identity of the *Ed. ictaluri* plasmid DNA fragments was confirmed by restriction endonuclease mapping and Southern analysis. Southern analysis was also used to screen for sequence similarity between the plasmid and chromosomal DNA of *Ed. ictaluri*. Furthermore, Northern analysis experiments were performed to test for expression of plasmid encoded genes in the bacterium. Finally, plasmid incompatibility experiments were used to cure *Ed. ictaluri* of native phasmid DNA.

Materials and Methods

I. Construction of a plasmid DNA library

A. Bacterial cultures

Bacteria used in this study included *Edwardsiella ictaluri* strain ATCC33202 and *Escherichia coli* strain RRIΔM15 (26). Because *Ed. ictaluri* requires a rich, complex medium, cultures of *Ed. ictaluri* were grown at 30°C in Brain Heart Infusion (BHI) broth (3). All *E. coli* cultures were grown at 37°C in Luria-Bertani (LB) broth. The plasmid vector, pTZ18U, was maintained in *E. coli* and used in all cloning procedures. Since pTZ18U contains an ampicillin resistant gene, cultures of *E. coli* harboring the vector or recombinant DNA molecules were grown in the presence of 100 μg/ml ampicillin.

B. Preparation of competent *E. coli* and *Ed. ictaluri*

The following method was modified from Maniatis et. al (17). *Escherichia coli* RR1ΔM15 was cultured in 50 ml of LB broth at 37° C until OD_{600} =0.3 was achieved. The culture was then cooled on ice for 5 min, and the cells collected by centrifugation at 1100 xg for 10 min. The supernatant was discarded and the cells were resuspended in 20 ml of 100 mM $CaCl_2$ and then incubated on ice for 30 min. The cells were collected again by centrifugation at 1100 xg for 10 min, resuspended in 5 ml of 100 mM $CaCl_2$, and stored at 4°C.

Edwardsiella ictaluri and *E. coli* cells were also made competent by a method modified from Hanahan et. al. (7). One ml of overnight broth cultures of *Ed. ictaluri* and *E. coli* RR1ΔM15 were used to inoculate 50 ml of BHI broth and 50 ml of LB broth,

respectively (7). The broths were incubated at the appropriate growth temperature for 3 h until OD_{600} =0.3 was achieved. The cultures were then placed on ice for 10 min. Cells were collected by centrifugation at 4300 xg for 6 min (Sorvall RC-5C Superspeed Centrifuge). The supernatant was drained and the pellet was resuspended in 20 ml of TFB (45 mM $MnCl_2$-$4H_20$, 10 mM $CaCl_2$-$2H_20$). The cells were placed on ice for 10 min and then recentrifuged at 4300 xg for 6 min. The supernatant was discarded and the pelleted cells were resuspended in 5 ml of TFB. DMSO was added to 3.5 %, the cells were swirled, and then placed on ice for 5 min. Then 181 μl of a 750 mM stock solution of 2-mercaptoethanol was added and the cells were incubated on ice for 10 min. An additional 175 μl of DMSO was added and the cells were incubated on ice for 5 additional min. Competent cells of *Ed. ictaluri* and *E. coli* were stored at 4°C. All competent cells were tested for transformation efficiency using the cloning vector, pTZ18U (17).

C. Isolation of plasmid DNA

Plasmid DNA was isolated from bacterial cells by the alkaline-hydrolysis technique and then purified by isopycnic ultracentrifugation through cesium chloride (17). Bacterial cells were collected from overnight 500 ml broth cultures by centrifugation at 4700 xg for 6 min (Sorvall RC- 5C Plus Superspeed Centrifuge). The supernatant was discarded and the cell pellet was resuspended in 10 ml of cold solution I (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 8.0, 25 mM glucose). Following a 5 min incubation at room temperature, cells were lysed in 20 ml of solution II (1% SDS, 0.2 N NaOH). The lysate was rocked gently for 5 min at room temperature before the addition of 15 ml of 3 M sodium acetate (pH 4.8). The solution was thoroughly mixed and placed on ice for 10

min. Cellular debris was removed by centrifugation at 13,000 xg for 10 min. The supernatant was transferred to a clean tube and the DNA precipitated by the addition of 27 ml of isopropanol. After a 15 min incubation at room temperature, the precipitate was collected by centrifugation at 15,000 xg for 10 min. The supernatant was then drained and the pellet allowed to air dry. The dried plasmid DNA was resuspended in 3 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

The plasmid DNA was separated from contaminating chromosomal DNA, RNA and protein by the addition of 3 g cesium chloride and 150 μl of a 10 mg/ml stock solution of ethidium bromide followed by ultracentrifugation at 1.8 X 10^7 xg for 18 h at 20° C (Sorvall Ultra Pro 80 Ultracentrifuge). Using a hand held UV-illuminator, the lower band representing covalently closed circular plasmid DNA was removed by puncturing the side of the gradient tube with a needle and drawing the band into a syringe. To extract the ethidium bromide, the plasmid DNA was added to an equal volume of isoamyl alcohol, mixed and centrifuged at 400 xg for 3 min (Sorvall RC-5C Plus Superspeed Centrifuge). The upper layer of isoamyl alcohol and ethidium bromide was discarded and the extraction with fresh isoamyl alcohol was repeated until all ethidium bromide was removed from the DNA sample. After the final extraction, the plasmid DNA was placed in dialysis tubing and dialyzed overnight with gentle agitation against TE buffer. The purified DNA was concentrated by alcohol precipitation, resuspended in 500 μl TE buffer and stored at - 20° C. The concentration of DNA was determined by UV-spectrometry at 260 nm using a Hitachi model 100-10 spectrophotometer. Purity of the DNA was confirmed by agarose gel electrophoresis (Section II-B).

D. Agarose gel purification of *Ed. ictaluri* plasmid DNA

The pCL 1 plasmid of *Ed. ictaluri* was isolated and purified from an agarose gel by a standard electroelution procedure (17). A 10 μg aliquot of *Ed. ictaluri* plasmid DNA was linearized by treatment with 20 units of Eco RI at 37°C for 2 h. The linearized plasmids were separated by agarose gel electrophoresis (Section II-B). The segment of agarose containing the upper 5.7 Kb band (pCL 1) was separated and placed in dialysis tubing containing TAE buffer. The dialysis tubing containing the agarose gel segment was then placed in the electrophoresis apparatus and electrophoresed at 100 V until the pCL 1 plasmid had migrated out of the gel and into the dialysis tubing. The TAE buffer now containing the pCL 1 plasmid DNA was removed from the dialysis tubing, alcohol precipitated and resuspended with 10 μl of TE buffer. Resuspended plasmid DNA was stored at -20°C.

E. Isolation of total cellular DNA from *Ed. ictaluri*

Isolation of total cellular DNA from *Ed. ictaluri* consisted of collecting cells from a 50 ml BHI broth culture by centrifugation at 4300 xg for 6 min (Sorvall RC-5C Superspeed Centrifuge). Cells were lysed in 1 ml of a solution containing 5% SDS and 0.1 M EDTA, prepared in 50 mM Tris (pH 8.0). DNA was precipitated by the addition of 100 μl of 3 M sodium acetate, pH 4.8, and 2 ml of isopropanol (31). A glass rod with a hook was used to spool the DNA from the solution. The DNA was placed in a microfuge tube, allowed to air dry, resuspended in 500 μl TE buffer and stored at 4°C. The concentration of total cellular DNA was determined by UV-spectrometry using a Hitachi model 100-10 spectrophotometer.

F. Isolation of RNA from *Ed. ictaluri*

A standard phenol extraction procedure was used to purify RNA from cultures of *Ed. ictaluri* (17). *Edwardsiella ictaluri* were grown in 5 ml of BHI broth overnight at 30°C and collected by centrifugation at 1100 xg for 15 min (Sorvall RC-5C Plus Superspeed Centrifuge). Cells were resuspended in 300 μl of SSE buffer (0.02 M sodium acetate, pH 5.5, 0.5% SDS, and 1mM EDTA) and then lysed by the addition of 300 μl phenol that had been previously equilibrated with 0.02 M sodium acetate pH 5.5. The solution was gently rocked at 60°C for 10 min. The tubes were then centrifuged at 1.5 X 10^4 xg (Sorvall RMC-14 Microcentrifuge) for 15 min and the aqueous layer removed to a clean microfuge tube. The aqueous layer was extracted once more with an equal volume of Tris- buffered phenol and centrifuged as before. The aqueous layer containing the RNA was removed to a clean microfuge tube and the RNA precipitated by the addition of 900 μl of ethanol and subsequent incubation on ice for 30 min. The precipitated RNA was collected by centrifugation at 1.5 X 10^4 xg for 15 min and then resuspended in 300 μl of SSE buffer. The RNA was precipitated with ethanol two more times. After the final precipitation, the RNA was resuspended in 10 μl of dH_20 and stored at - 20 °C.

II. Construction of a DNA library using plasmid DNA from *Ed. ictaluri*

A. Restriction endonuclease digestion of DNA

Restriction endonucleases used to digest the DNA were selected based on restriction endonuclease maps of *Ed. ictaluri* plasmid DNA and the plasmid vector pTZ18U (Figure 1). All restriction endonucleases were obtained from Promega Corporation (Madison, WI). The digests were performed at 37°C using either 6 μg of

Ed. ictaluri plasmid DNA or 2 μg of pTZ18U for 4 h in the appropriate digestion buffer (Promega Corporation) and 10 units of each restriction endonuclease. After digestion, the samples were placed at -20°C until used in the ligation procedure. Complete digestion was confirmed by agarose gel electrophoresis of 1 μl of each digest.

B. Agarose gel electrophoresis of DNA

Agarose gels were prepared by dissolving 0.3 g of agarose in 30 ml of TAE buffer (0.04 M Tris-acetate, 1 mM EDTA) by heating to 100°C. After the agarose solution cooled, 1 μl of a 10 mg/ml stock solution of ethidium bromide was added to the molten agarose and then poured into an agarose gel forming apparatus (Horizon 58 Electrophoresis System, Gibco BRL, Life Technologies, Inc., Gaithersburg, MD). After the gel solidified, the comb was removed and 200 ml of TAE buffer containing 10 μl of 10 mg/ml ethidium bromide was added to a depth of 1 mm over the gel. DNA samples were mixed with 6 X gel-loading buffer (0.25 % bromphenol blue 0.25 % xylene cyanol, 30 % glycerol) and loaded into the wells of the submerged gel. Electrophoresis of the DNA gels was performed at 100 V for appropriate length of time. The DNA fragments were visualized by UV-transillumination.

C. Ligation of plasmid DNA fragments from *Ed. ictaluri* into the plasmid vector pTZ18U

Three separate ligations of *Ed. ictaluri* plasmid DNA into the plasmid vector, pTZ18U were performed as shown in Table 1. Each ligation (A, B, C) contained 6 μg of *Ed. ictaluri* DNA and 2 μg of the appropriately digested pTZ18U. The DNA's were concentrated by alcohol precipitation and the precipitate dried. Each ligation reaction was

resuspended in 18 μl of TE buffer, 2 μl 10X ligation buffer (Promega) and 3 units of T4 DNA ligase (Promega) followed by overnight incubation at 4°C.

D. Transformation of *E. coli*

The following transformation method was modified from Maniatis et. al.(17). The transformation protocol consisted of 100 μl competent *E. coli* RRIΔM15 and the ligation reactions from above (Table 1). The transformation reactions were incubated on ice for 45 min, heat shocked at 37°C for 30 s and then incubated at room temperature for 1 min followed by the addition of 300 μl of LB broth. Cells were then incubated at 37°C for 30 min and 100 μl of each sample were plated onto MacConkey agar plates containing 100 μg/ml ampicillin and 0.05 mM IPTG. For each transformation reaction, three replicate plates were inoculated and incubated at 37°C overnight.

III. Characterization of recombinant DNA clones of *Ed. ictaluri* plasmid DNA

A. Restriction endonuclease mapping of recombinant clones.

After observation of the plates, white colonies containing recombinant molecules from each transformation were selected to inoculate nutrient broth containing 100 μg/ml ampicillin. Following an overnight incubation at 37°C, a small scale plasmid isolation procedure was used to purify the recombinant plasmids from each culture. The purified DNA was then subjected to electrophoresis.

Digestion of the recombinant clones was carried out at 37°C for 2 h. Each digest contained 10 μl of DNA, 17 μl of TE buffer, 3 μl 10X digestion buffer, and 10 units of the appropriate restriction endonuclease (17). Digests were alcohol precipitated, air dried, and resuspended in 4 μl of TE buffer. Electrophoresis of digests was performed

on 1 % agarose gels and visualized by UV-illumination as described above. The Lambda Hind III marker (Gibco BRL, Life Technologies, Inc,. Gaithersburg, MD) was used as a molecular weight standard.

Clones selected for further characterization were individually cultured in 500 ml LB broth containing 100 μg/ml ampicillin. Plasmid DNA was isolated and purified by cesium chloride ultracentrifugation as described above (Section I-C).

Restriction endonuclease mapping experiments followed by Southern analysis were used to determine whether the cloned fragments originated from pCL 1 or pCL 2. Digestion of 1 μg of cloned DNA's with 0.5 μl of the appropriate restriction endonucleases were conducted at 37°C for 2 h. After digestion, the cloned DNA fragments were alcohol precipitated as described above, and analyzed by electrophoresis on 1 % agarose gels (Section II-B).

B. Labeling DNA for use as genetic probes

Edwardsiella ictaluri plasmid DNA and cloned plasmid DNA fragments of *Ed. ictaluri* were labeled as genetic probes and used in the following Southern and Northern procedures. A 0.5 μg aliquot of DNA was labeled by primer extension with biotinylated nucleotides as described by the Gibco BRL BluGene Kit (Life Technologies, Gaithersburg, MD).

C. Southern analysis of the DNA fragments

Southern analysis of the DNA fragments was performed to confirm the identity of the cloned fragments and to determine if any of the cloned DNA fragments contained sequences complementary to chromosomal DNA of *Ed. ictaluri*. After electrophoresis of

the DNA fragments, the DNA was partially depurinated by soaking the gels in 0.25 M HCl for 5 min. The DNA was then denatured by soaking in 0.4 N NaOH for 15 min. A nylon filter (Magna Graph Nylon Transfer Membrane, Micron Separations Inc. Westboro, MA) was cut to the size of the gel and wetted in dH_2O along with 2 sheets of 3 MM Whatman paper also cut to the size of the gel. A prewetted 3 MM Whatman paper wick was placed on an inverted petri dish in the bottom of a tray containing 0.4 N NaOH. The gel was inverted and placed on the wet wick followed by the nylon filter and the 2 sheets of 3 MM Whatman paper. A stack of paper towels cut to the approximate size of the gel was then placed on top of the 3 MM Whatman paper. DNA was allowed to transfer overnight at room temperature by capillary action. After the transfer was complete, the nylon filter was removed, briefly rinsed in 2X SSC (3 M sodium chloride, 0.3 M sodium citrate) and allowed to air dry.

The dried nylon filters were prehybridized in a solution of 5X SSC, 25 mM sodium phosphate (pH 6.5), 0.5% SDS, 0.5 % powdered milk and 5X Denhardt's solution for 2 h at 56° C. The prehybridization solution was removed and replaced with a hybridization solution (5X SSC, 25 mM sodium phosphate, pH 6.5, 0.5 % SDS, 0.5% powdered milk and 1X Denhardt's) containing 100 ng/ml labeled genetic probe (Section III-B) which had been denatured at 100° C for 10 min. The filters were hybridized overnight at 56° C (Gibco BRL BluGene Detection Kit, Life Technologies, Gaithersburg, MD).

After hybridization, filters were washed twice in 2X SSC containing 0.1% SDS for 3 min at room temperature, twice in 0.2X SSC and 0.1% SDS for 3 min at room

temperature, and twice in 0.16X SSC and 0.1% SDS for 15 min at 50°C. Filters were then rinsed briefly in 2X SSC and placed in Buffer 1 (0.1 M Tris-HCl, ph 7.5, 0.15 M NaCl) for 1 min at room temperature. Filters were then incubated for 1 h at 65°C in Buffer 2 (3 g BSA/100 ml Buffer 1). The labeled probe was detected by a strepavidin-alkaline phosphatase conjugate and visualized with the chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium as described in the Gibco BRL Hybridization and Detection manual (Life Technologies, Gaithersburg, MD).

D. Northern analysis of RNA from *Ed. ictaluri*

Possible encoded genes of the pCL 1 plasmid was investigated using Northern analysis of RNA isolated from *Ed. ictaluri*. Formaldehyde agarose gels were prepared by dissolving 0.45 g of agarose in 18.6 ml dH_20 by heating to 100 °C. The solution was allowed to cool to 60° C before the addition of 6 ml 5X gel running buffer (0.2 M 3-[N – Morpholino] propane-sulfonic acid pH 7.0, 50 mM sodium acetate, 5 mM EDTA pH 8.0) and 18 % formaldehyde. The gel solution was mixed thoroughly and poured into a gel forming apparatus (Horizon 58 Electrophoresis System, Gibco BRL, Life Technologies, Inc., Gaithersburg, MD). After the gel solidified, the comb was removed and 5 X gel running buffer was added to a depth of 1 mm over the gel. Ten microgram samples of RNA were denatured in 20 μl of a solution of 0.2 M 3-[N-Morpholine] propane-sulfonic acid pH 7.0, 50 mM sodium acetate, 5 mM EDTA pH 8.0, containing 18 % formaldehyde and 54 % formamide. Once denatured the samples of RNA were mixed with a RNA loading buffer (50 % glycerol, 1 mM EDTA, 0.4 % xylene cyanol, and 0.4 % bromophenol blue) and loaded into the wells of the submerged gel. Electrophoresis of the

RNA gels were performed at 40 V at 4°C for an appropriate length of time.

After electrophoresis, RNA gels were transferred to nylon membranes using 50 mM NaOH by the procedure described above but omitting the depurination step with 0.25 M HCl (Section III-C). Hybridization with the appropriate genetic probes was performed as described above (Sections III-C).

E. Transformation of *Ed. ictaluri* cells

Plasmid incompatibility experiments were performed using the recombinant DNA molecules characterized above to transform *Ed. ictaluri* made competent by the TFB procedure (Section I-B) (7). The transformation reaction consisted of 100 μl of competent *Ed. ictaluri* cells in chilled microfuge tubes and 1 μg of plasmid DNA. The reactions were incubated on ice for 30 min, heat shocked at 30°C for 90 s and then incubated on ice for 1 min. Following the addition of 800 μl of BHI broth, the reactions were incubated at 30°C for 1 h. One hundred microliters of each sample were plated on BHI agar plates containing 100 μg/ml ampicillin followed by incubation at 30°C for 48 h. Plates containing colonies were counted for transformation efficiency.

One colony from each of the successful transformation reactions was cultured in 50 ml of BHI broth containing 100 μg/ml ampicillin. Plasmid DNA was then isolated from each culture by the alkaline hydrolysis procedure (Section I-C). The purified DNA was then subjected to digestion with restriction endonucleases, electrophoresed and analyzed by Southern techniques using pTZ18U as the genetic probe (Sections III-C).

Results

A total of 47 transformants were obtained from the cloning procedures. Twenty four of these transformants were randomly selected and analyzed by restriction endonuclease mapping experiments. The resulting DNA fragments were electrophoresed on 1 % agarose gels, and visualized by UV-illumination. From the banding patterns observed on the gels the twenty-four clones could be grouped into four unique classes. A representative isolate was selected from each class for further study. Figure 2A shows the banding patterns obtained for the four classes of recombinants. The four *Ed. ictaluri* plasmid DNA clones characterized in this study were designated A10, B1, B10, and C13. The letter designation corresponds to the respective ligation reactions (Table 1). Restriction endonuclease mapping of the four clones were then performed to begin to determine if the cloned fragments originated from pCL 1 or pCL 2. During the construction of the recombinant clones, the restriction endonuclease Bgl II was used to cleave the pCL 1 plasmid while Bam HI was used to cleave the cloning vector pTZ18U (Table 1). The ligation of a DNA fragment cleaved with Bgl II to another DNA molecule cleaved with Bam HI will result in the loss of the recognition sequence for both Bgl II and Bam HI. Therefore, recombinant clones containing the Eco RI/ Bgl II fragment had to be digested with the restriction endonuclease, Xba I whose recognition site was positioned near the Bam HI cloning site on the pTZ18U vector (Figure 1). Restriction endonucleases used to digest the clones were:

Clones	Restriction Endonuclease
A10	Eco RI, Xba I
B1	Eco RI
B10	Eco RI, Sac I
C13	Eco RI, Sac I, Bgl II

The resulting DNA fragments were analyzed by agarose gel electrophoresis (Figure 2A). All clones contained fragments originating from pCL 1. The approximate sizes of the cloned fragments are A10=1.3 kb Eco RI/ Bgl II fragment, B1=5.7 kb Eco RI fragment, B10=3.4 kb Eco RI/ Sac I fragment, and C13=4.7 kb Sac I/ Bgl II fragment (Figure 3A and 3B). Southern analysis was then used to confirm the identity of the recombinant molecules. Only DNA bands representing pCL 1 fragments hybridized to the labeled pCL 1 gene probes (Figure 2B).

Next, Southern analysis was used to test for regions of homology between pCL 1 and chromosomal DNA of *Ed. ictaluri*. Total cellular DNA was purified from an overnight 50 ml *Ed. ictaluri* culture and digested with the restriction endonuclease Eco RI. Electrophoresis on 1 % agarose gels was then performed to separate the plasmid DNA from the chromosomal DNA (Figure 4A). After transferring to a nylon membrane, Southern analysis was performed using genetic probes representing the pCL 1 plasmid, the recombinant clones pA10, pB1, pB10, pC13 and the cloning vector pTZ18U. No hybridization was observed between any of the genetic probes of pCL 1 and chromosomal DNA (Figure 4B).

In addition, it was deemed important to determine if the recombinant clones

contained a gene segment that is expressed in *Ed. ictaluri*. Northern analysis was conducted using genetic probes representing the pCL 1 plasmid and the recombinant clones pA10, pB1, pB10, and pC13. RNA was isolated from an overnight culture of *Ed. ictaluri* by the phenol extraction method and separated on formaldehyde agarose gels, transferred to a nylon filter and hybridized with the genetic probes. A faint smear representing a heterogenous mixture of different sized RNAs was observed on the gel (Figure 5A). When the RNA was subjected to Northern analysis, hybridization was not observed for any of the recombinant DNA genetic probes nor the pCL 1 gene probe.

Since no hybridization was detected between the RNA from *Ed. ictaluri* and the pCL 1 probe, the sensitivity of the hybridization assay was determined. Serial two fold dilutions of plasmid DNA purified from *Ed. ictaluri* were electrophoresed on a 1 % agarose gel, transferred to a nylon membrane, and hybridized to labeled pCL 1 plasmid DNA. The genetic probe was able to detect 7.5 ng of plasmid DNA (Figure 5B).

Because plasmid curing has been used to document the importance of plasmid DNA to the virulence properties of bacteria, a plasmid incompatibility experiment was performed using the recombinant plasmids to transform *Ed. ictaluri*. The standard calcium chloride procedure was first attempted for the transformation of *Ed. ictaluri*, but it was found that the $CaCl_2$ treatment killed the bacterium. A different protocol using TFB buffer was then tested and the *Ed. ictaluri* were found to survive the treatment (7). After *Ed. ictaluri* cells were made competent with TFB, a transformation reaction was performed with each of the recombinant clones and the cloning vector, pTZ18U. Only the recombinant clones pA10 and pB10 were successful in transforming *Ed. ictaluri*. The

efficiency of the transformation of TFB competent *Ed. ictaluri* with recombinant pA10 and pB1 was 2700 cfu/μg and 18 cfu/μg respectively (Table 2). Transformants of *Ed. ictaluri* with pA10 and pB1 recombinant DNAs were selected and cultured individually in BHI containing 100 μg/ml ampicillin. A plasmid DNA isolation procedure was used to isolate DNA from each transformant. The resulting DNA preparations were then analyzed by restriction endonuclease digestion with Eco RI, electrophoresis through a 1 % agarose gel and Southern analysis with the pTZ18U gene probe. Electrophoresis of the DNA obtained from both the pA10 and pB1 transformed cells, revealed one faintly staining band (Figure 6). The pTZ18U genetic probe, hybridized to the faint band.

Previous studies have shown that *E. coli* can be successfully transformed by either the $CaCl_2$ or TFB procedures (7, 17). In my study, *E. coli* RR1ΔM15, were used as a control for the comparison of transformation efficiencies obtained for *Ed. ictaluri.* All recombinant clones and pTZ18U successfully transformed *E. coli* RR1Δ M15 when the cells were made competent with $CaCl_2$. Interestingly, only recombinant clones pA10, pB10 and the cloning vector pTZ18U were successful in transforming TFB treated *E. coli* RR1Δ M15. The transformation efficiencies for these experiments are shown in Table 3. Transformation efficiencies of $CaCl_2$ treated *E. coli* cells were 5525 cfu/μg for pA10, 1052 cfu/μg for pB1, 1272 cfu/μg for pB10, 400 cfu/μg for pC13 and 288 cfu/μg for pTZ18U. Transformation efficiencies for the TFB treated *E. coli* RR1ΔM15 were 1206 cfu/μg for pA10, 108 cfu/μg for pB10 and 18 cfu/μg for pTZ18U.

Discussion

Since the discovery of *Edwardsiella ictaluri* in 1976, much of the research has focused on developing effective methods for identifying isolates of *Ed. ictaluri* from infected channel catfish. These identification methods involve the examination of plasmid DNA profiles and biochemical characterization of *Ed. ictaluri* (15, 16, 20, 25, 36). In addition, research has documented the histopathology and pathogenic process of enteric septicemia (19, 30). The mechanism or mechanisms by which *Ed. ictaluri* infects and damages its host have not been demonstrated. However, *Ed. ictaluri* is known to be resistant to 20 % serum (24). Serum resistance is a known virulence factor for other Gram negative bacteria and is a property encoded by plasmid DNA in these bacteria (38). This focused on obtaining a recombinant DNA library of plasmids purified from *Ed. ictaluri*. The plasmid DNA library was characterized by Southern and Northern blotting experiments as well as analysis of the recombinant clones the ability to cure the native plasmid DNA from *Ed. ictaluri*. As a result of constructing this library, it is hoped that future experiments will identify genes encoded by the plasmid DNA of *Ed. ictaluri* and the role these genes play in virulence.

Construction of the recombinant DNA library was accomplished using multiple restriction endonucleases to obtain overlapping fragments of the plasmid DNA. Based on the restriction endonuclease maps, digestion with these restriction endonucleases should have resulted in obtaining five different classes of recombinant molecules (Figure 1). However, only three of the five distinct recombinant patterns were obtained. Interestingly

all recombinant clones contained pCL 1 DNA and no clones were identified for pCL 2. Furthermore, a recombinant clone was isolated and found to harbor a full length copy of pCL 1 (Figure 3A and 3B). Restriction endonuclease maps of the recombinant clones as well as pCL 1 and pCL 2 DNA's were consistent with the data published by Lobb et. al (16). These data are significant since I used the same strain of *Ed. ictaluri* in the construction of my DNA libraries as described by Lobb et. al. (16). Positive hybridization of a pCL 1 gene probe to each of the recombinant clones confirmed that these clones did originate from the pCL 1 plasmid of *Ed. ictaluri* (Figure 2B).

In a separate investigation, Newton et. al. (1988) used a single restriction endonuclease, Eco RI, to construct full length clones of both plasmids from *Ed. ictaluri* (20). In my study, the use of multiple restriction endonucleases allowed us to clone overlapping fragments of one of the plasmids, pCL 1. However, my experiments were unsuccessful in generating any recombinant clones of pCL 2. Research using clones containing overlapping DNA fragments will allow the isolation and identification of genes encoded by the plasmid DNA as well as map these genes to certain locations on the plasmid DNA.

Earlier work has shown that no sequence homology exists between the chromosomal and plasmid DNA of *Ed. ictaluri* (23). In support of these earlier investigations, I also screened for homologous segments of DNA between the bacterial chromosome and pCL 1. Southern analysis using pCL 1 and the recombinant plasmids as genetic probes, failed to demonstrate any homology between the chromosomal DNA of *Ed. ictaluri* and pCL 1. This confirms the plasmid DNA of *Ed. ictaluri* to be distinct from

total cellular DNA and therefore may serve some purpose to the organism.

In addition, previous research addressing the possibility of homologous sequences shared between pCL 1 and pCL 2 have been contradicting (15, 32). In my investigation the recombinant DNA clones were analyzed for sequences homologous with pCL 2. Surprisingly homology was demonstrated between the recombinant clones and the pCL 2 plasmid. Furthermore, when pCL 1 was purified and used as a probe, homology was again demonstrated with pCL 2. Experiments are being designed to isolate and identify the homologous sequences.

Next, I decided to demonstrate expression of genes encoded on pCL 1 and identify recombinant clones containing these genes. Northern analysis was performed, on total cellular RNA isolated from *Ed. ictaluri*, using pCL 1 plasmid DNA and the recombinant clones as probes. Following electrophoresis, the RNA was visualized by staining with ethidium bromide and UV-illumination. A heterogenous mixture of different sized RNA molecules which are representative of numerous transcripts from the genes of *Ed. ictaluri* were visualized (Figure 5A). None of my genetic probes, pCL 1 nor any of the recombinant molecules, hybridized to the RNA from *Ed. ictaluri*. The absence of any hybridization could be explained if the plasmid encoded genes are expressed only under specific environmental conditions. All *Ed. ictaluri* cultures were grown in nutrient rich media and at the optimal growth temperature for the bacterium. Since the *Ed. ictaluri* cells were not under any environmental stress, it is possible that expression of the plasmid encoded genes only occurs in response to environmental stress. Environmental stress has been shown to be a regulator of the plasmid encoded gene, *gsr* A, in *Yersinia*

enterocolitica. The *gsr* A gene product is needed by the bacterium to survive within phagocytic cells by resisting the microbicidal mechanisms of macrophages (37). Previous studies have demonstrated that *Ed. ictaluri* will enter a dormant phase in the macrophage of the catfish or in pond mud when environmental temperatures move outside the optimal growth range (4), and that *Ed. ictaluri* grows poorly in nutrient deficient medium (3). Future research into the coding capacity of the plasmid DNA will include growth of the bacterium under conditions that are representative of temperatures and available nutrients found in the catfish and pond water.

Since no hybridization was evident in the Northern analysis experiments, the sensitivity of the hybridization reaction was investigated. Serial dilutions of plasmid DNA from *Ed. ictaluri* were electrophoresed through a 1 % agarose gel, transferred to a nylon membrane, and hybridized with the pCL 1 probe. The procedure was capable of detecting 7.5 ng of DNA. At this level of detection, the cells need to be expressing their plasmid encoded genes at a 10-fold higher concentration than the chromosomal genes. In a attempt to correct for this level of detection the Northern blot analysis were repeated using 10 μg of RNA. Again no hybridization was observed with any of the genetic probes (data not shown). In the future, experiments will be designed to identify the growth conditions needed for optimal expression of the plasmid encoded genes of *Ed. ictaluri*.

Since no hybridization was detected in my Northern analysis, the recombinant clones were tested for ability to cure the native plasmid DNA from *Ed. ictaluri*. The successful curing of the plasmid DNA from *Ed. ictaluri* by any method has not been demonstrated. However, an unsuccessful attempt using growth elevated temperatures has

been reported (16). In my study, I chose to rely on plasmid incompatibility to cure the plasmids from *Ed. ictaluri*. Each of the recombinant clones were transformed into *Ed. ictaluri* by a TFB procedure modified slightly from that described by Hanahan et al (7).

Interestingly, two of the pCL 1 recombinant clones (pA10 and pB1) successfully transformed and subsequently cured *Ed. ictaluri*. Confirmation of transformation and curing was demonstrated in 3 ways: 1) the growth of transformed cells in the presence of ampicillin, 2) detection of pTZ18U DNA in the transformed cells, and 3) the absence of pCL 1 and pCL 2 DNAs. The plasmid DNA observed in the transformed cells was approximately 9 kb as determined by agarose gel electrophoresis. Since this plasmid is different in size when compared with either pCL 1, pCL 2, pA10, or pB1. It is believed that genetic recombination occurred between the recombinant plasmid and pCL 1. Since native plasmid DNA from *Ed. ictaluri* would have migrated with sizes approximately 5.7 kb (pCL 1) and 4.9 kb (pCL 2) on the agarose gel and no DNA bands were observed in these regions, the pCL 1 and pCL 2 plasmid have been cured from the *Ed. ictaluri* cells.

The transformation efficiencies of *Ed. ictaluri* with pA10 and pB1 are listed in Table 2. The difference in transformation efficiencies of the two clones may be explained by the size of the plasmid DNA, 4.1 kb and 9.5 kb respectively. Smaller DNA molecules transform at higher efficiencies than larger DNAs. The absence of transformation of the other recombinant clones may be due to a recently described restriction-modification system in *Ed. ictaluri* (3). Many bacteria contain restriction-modification systems to protect themselves from invading DNA molecules as in bacteriophage infections.

Restriction-modification systems work by either destroying foreign DNA by restriction endonuclease digestion or protecting DNA by methylation of restriction endonuclease sites (21). The two clones, pB10 and pC13, that did not transform *Ed. ictaluri* share a common region (Eco RI-Sac I fragment) which may contain the target sequence for the restriction system. Since pB1 also contains this segment yet it does transform *Ed. ictaluri*, then the additional Bgl II-Sac I region unique to pB1 is possibly responsible for modification. Interestingly, pA10 which lacks both the Eco RI-Sac I segment and the Bgl II-Sac I segment and yet was clearly able to transform *Ed. ictaluri* and cure the native plasmid DNA. Since pA10 was able to transform *Ed. ictaluri* and cure the cell of native plasmid DNA, the pA10 clone is believed to contain the origin of replication of pCL 1.

Escherichia coli RR1ΔM15, was used as a control to compare transformation efficiencies. All of the recombinant clones successfully transformed the *E. coli* when the standard $CaCl_2$ procedure was used. However, only three recombinant plasmids were able to transform the TFB treated *E. coli*. The efficiency of transformation procedures have been demonstrated to be effected by the concentration of calcium ions which are responsible for binding of the DNA to the cell surface (21). The calcium ions used in the $CaCl_2$ and TFB procedures were 100 mM and 10 mM respectively. Therefore, the TFB procedure may not contain enough calcium ions to effectively bind the DNA to the cell surface. This may explain why the transformation efficiency of the TFB procedures were lower than the $CaCl_2$ and the lack of transformation seen for some of the cloned DNA. It is important to note that treatment of *Ed. ictaluri* with 100 mM $CaCl_2$, the optimal concentration for transformation, resulted in killing *Ed. ictaluri*. Future research will alter

the concentration of calcium ions to increase the transformation efficiency of *Ed. ictaluri*.

As stated earlier, there have been no studies correlating the plasmid DNA of *Ed. ictaluri* with any virulence properties of the bacterium. Therefore this investigation focused on constructing a DNA fragment library representative of *Ed. ictaluri* plasmid DNA for characterization by molecular techniques. By cloning the plasmid DNA in fragments, discrete segments of the plasmid could be investigated for biological activity. Future research will focus on determining the nucleotide sequence for each of the cloned segments of DNA and the analysis of these sequences for open reading frames and other functional elements. Regions on the plasmids encoding for genes were not demonstrated in this study at optimal growth conditions. Further research needs to be conducted to determine if encoded genes of the pCL 1 plasmid are controlled by environmental stress. This may include growth of the bacterium in nutrient concentrations and at temperatures representative of conditions found in the catfish. Transformation of *Ed. ictaluri* cells with cloned DNA using plasmid incompatibility properties proved to be an important method of curing native plasmid DNA. It will also be important to determine if the plasmid-cured strains of *Ed. ictaluri* are pathogenic for catfish. If these new strains of *Ed. ictaluri* are nonpathogenic and retain their immunogenic properties then they can be considered for the development of a vaccine.

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Appendix

Table 1: Ligation of the restriction endonuclease digestion of *Ed. ictaluri* plasmid DNA and pTZ18U vector DNA.

Ligations	Insert *Ed. ictaluri* Digests	Vector pTZ18U Digest
A	Eco RI, Bgl II, Sac I	Eco RI Bam HI
B	Eco RI, Bgl II, Sac I	Eco RI, Sac I
C	Eco RI, Bgl II, Sac I	Bam HI, Sac I

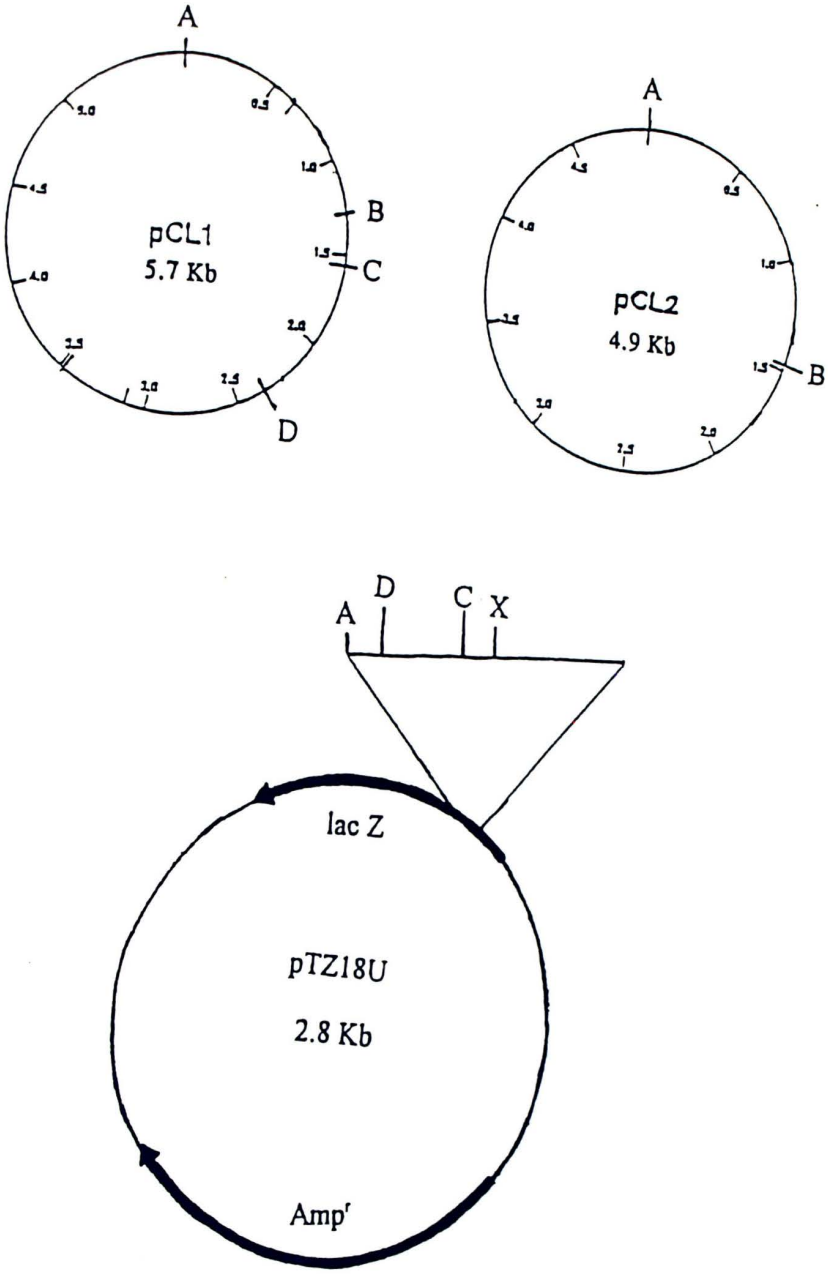

Figure 1: Restriction endonuclease maps of the *Edwardsiella ictaluri* plasmids, pCL 1 and pCL 2, and the cloning vector pTZ18U. Letters represent restriction endonucleases: A=Eco RI, B=Bgl II, C=Bam HI, D=Sac I, and X=Xba I. Lac Z refers to the region on the pTZ18U cloning vector that encodes for beta-galactosidase. Amp^r refers to the region that encodes for ampicillin resistance on the pTZ18U cloning vector.

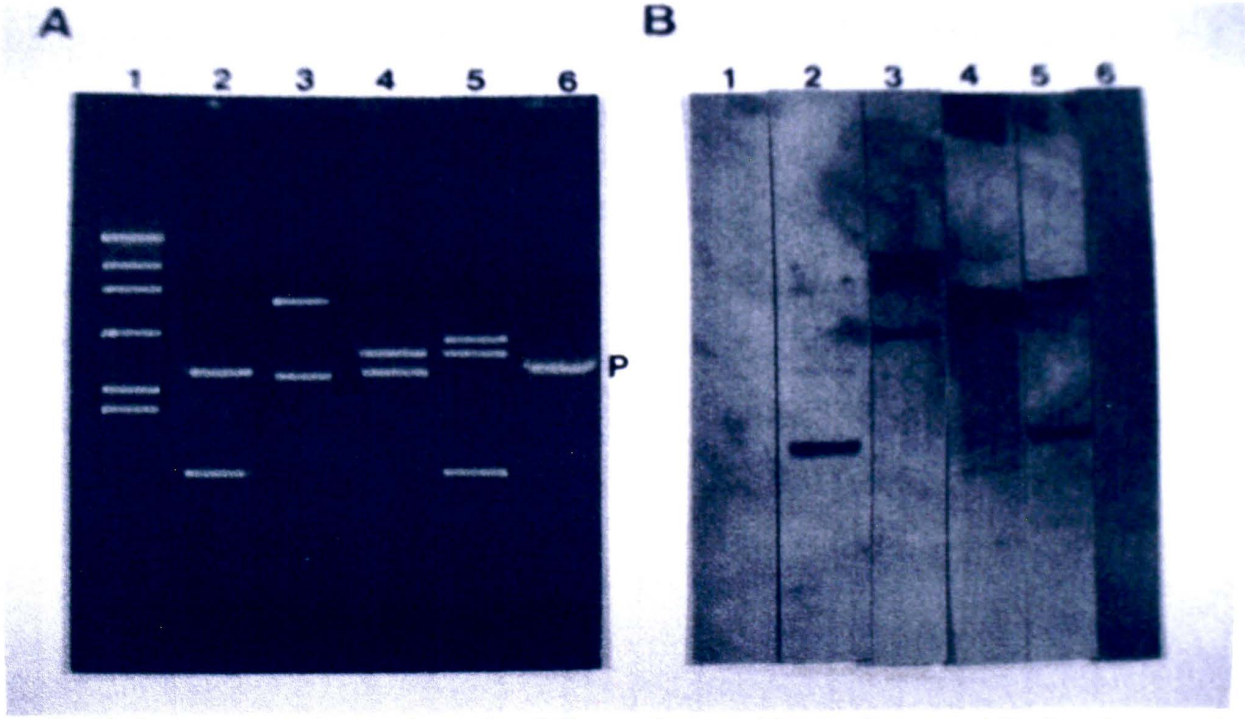

Figure 2: A) Agarose gel electrophoresis of digested recombinant clones and B) Southern analysis of digested clones with a labeled pCL 1 gene probe. A) and B) Lanes 1= Lambda Hind III marker, 2=pA10 digested with Eco RI and Xba I, 3=pB1 digested with Eco RI, 4=pB10 digested with Eco RI and Sac I, 5=pC13 digested with Eco RI, Sac I, and Bgl II, 6=pTZ18U cloning vector, also designated by the P. Approximate sizes of the Lambda Hind III marker are 23 kb, 9.4 kb, 6.5 kb, 4.3 kb, 2.3 kb, 2.0 kb, and 5.6 kb. Approximate size of clones pCL 1 fragments: A10=1.3 kb, B1=5.7 kb, B10=3.5 kb, C13=4.7 kb. pTZ18U=2.8 kb. B) All clones hybridized to the labeled gene representing the pCL 1 plasmid of *Ed. ictaluri*.

A

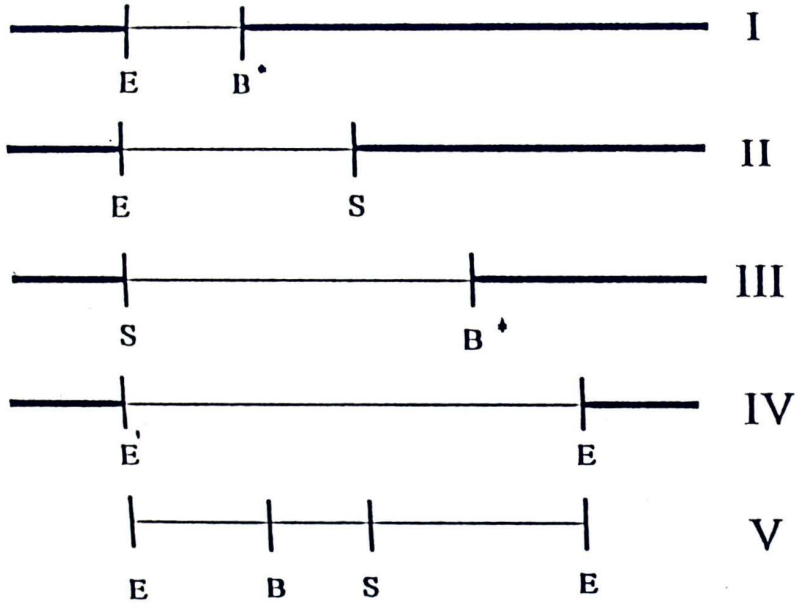

Figure 3A: Diagram of clones from *Edwardsiella ictaluri* plasmid pCL 1 showing size and restriction endonuclease cloning site. Roman numerals represent clones: I=A10, II=B10, III=C13, IV=B1 and V=pCL 1 plasmid. Letters represent restriction endonucleases: E=Eco RI, B=Bgl II, B*=Bgl II/Bam HI and S=Sac I. Thin line, ——— represents insert *Ed. ictaluri* plasmid DNA. Thick line, ━━━ represents vector pTZ18U DNA.

B

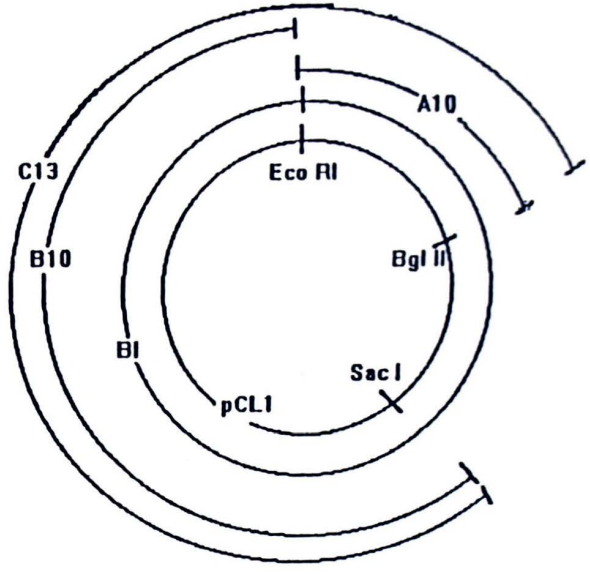

Figure 3B: Diagram of the pCL 1 plasmid (5.7kb) Showing the fragments cloned as A10=1.3kb, B1=5.7 kb, B10=3.5 kb, and C13=4.7 kb. Eco RI, Bgl II, and Sac I are the restriction endonucleases used to clone the fragments of the pCL 1 plasmid of *Ed. ictaluri*.

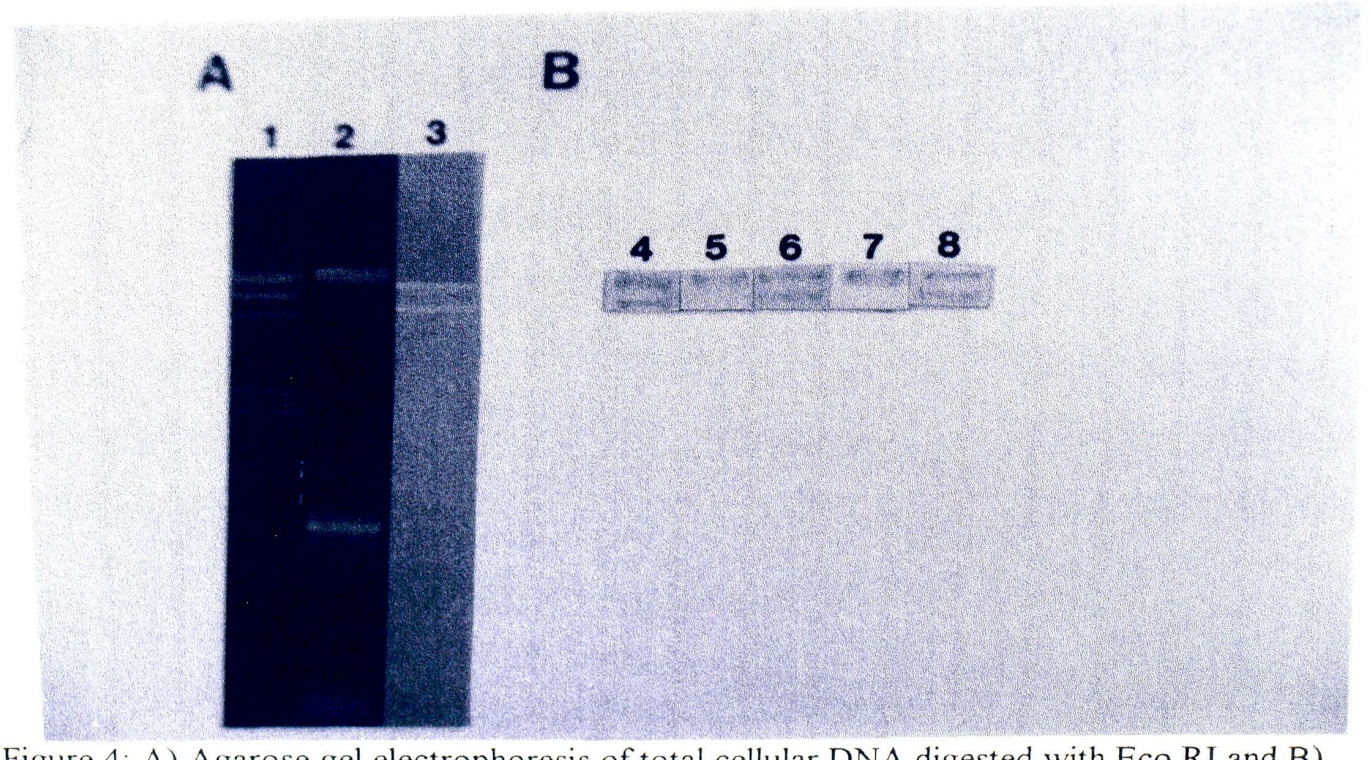

Figure 4: A) Agarose gel electrophoresis of total cellular DNA digested with Eco RI and B) Southern analysis of total cellular DNA with genetic probes representing pCL 1 plasmid and the fragment clones of pCL 1. Lane designation: A-1) Lambda Hind III marker, A-2) migration of the chromosomal DNA of *Ed. ictaluri*, A-3) migration of plasmid DNA of *Ed. ictaluri*, B-4) hybridization of pCL 1 probe with the pCL 1 plasmid, B-5) hybridization of pA10 gene probe with the pCL 1 plasmid, B-6) hybridization of pB1 gene probe with the pCL 1 plasmid, B-7) hybridization of pB10 probe with the pCL 1 plasmid, B-8) hybridization of pC13 probe with the pCL 1 plasmid.

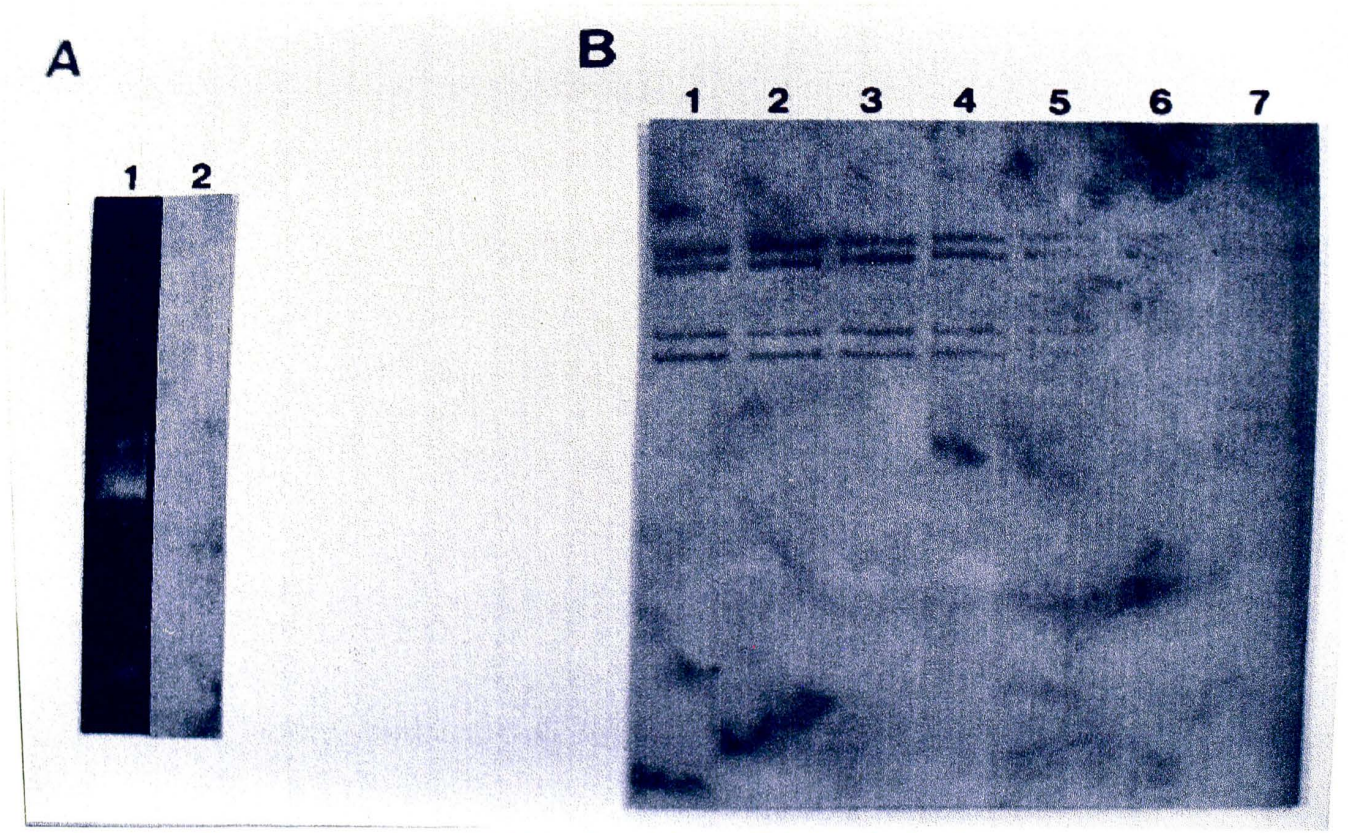

Figure 5: A-1) Electrophoresis of RNA isolated from *Ed. ictaluri*. Smear represents heterogenous mixture of different sized RNAs. A-2) Northern analysis of RNA gel. No hybridization of the RNA was detected with the pCL 1 gene probe. B) Southern analysis of 1:2 serial dilution of *Ed. ictaluri* plasmid DNA hybridized with the pCL 1 gene probe.

Table 2: The transformation efficiency of *Edwardsiella ictaluri* and *E. coli* RR1△M15 cells treated with 100 mM $CaCl_2$ and TFB.

	100 mM $CaCl_2$ (cfu/μg)	**TFB (cfu/μg)**
Edwardsiella ictaluri		
A10	0	2700
B1	0	18
B10	0	0
C13	0	0
pTZ18U	0	0
RR1△M15		
A10	5525	1206
B1	1052	0
B10	1272	108
C13	400	0
pTZ18U	288	18

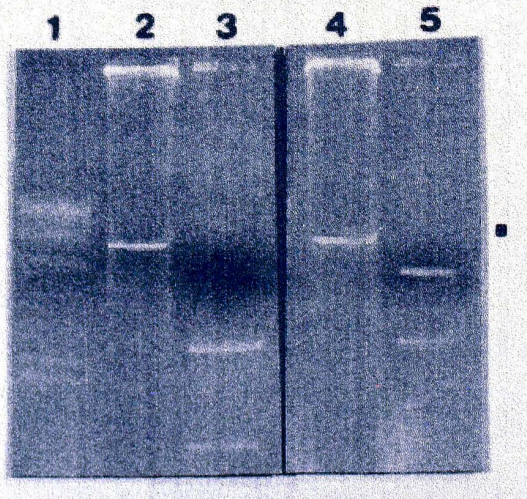

Figure 6: Agarose gel electrophoresis of transformed *Ed. ictaluri*. Lane designation : 1) Lambda Hind III marker, 2 and 4) 9.4 kb band designated by the dot, 3) recombinant clone pA10 digested with Eco RI and Xba I, 5) recombinant clone pB1 digested with Eco RI.

VITA

Contessa Morgan-Wood was born in Lexington, Kentucky on February 17, 1971. She attended elementary school in Trenton, Kentucky and graduated from Todd County Central High School, Elkton, Kentucky in May 1989. The following August, she attended Austin Peay State University and in August 1994, graduated with a Bachelor of Science degree in Biology. She then entered graduate school at Austin Peay State University where she will receive the degree of Master of Science in Biology in May 1997.

She is currently employed by Vanderbilt University Biomedical Center as a Research Assistant in the Infectious Disease department.