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Development of an Electrotransformation Technique for *Streptococcus iniae* and Preliminary Characterization of the Hemolysin Associated with this Bacterium

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DEVELOPMENT OF AN ELECTROTRANSFORMATION TECHNIQUE FOR *STREPTOCOCCUS INIAE* AND PRELIMINARY CHARACTERIZATION OF THE HEMOLYSIN ASSOCIATED WITH THIS BACTERIUM

by

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ABSTRACT

DEVELOPMENT OF AN ELECTROTRANSFORMATION TECHNIQUE FOR *STREPTOCOCCUS INIAE* AND PRELIMINARY CHARACTERIZATION OF THE HEMOLYSIN ASSOCIATED WITH THIS BACTERIUM

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Streptococcus iniae is a gram-positive organism responsible for causing disease in both freshwater as well as saltwater fish. Every year fisheries lose fish by the tons due to diseases caused by this organism. In 1991, the first reported human case of disease associated with this organism was described. Since this initial case, there have been numerous other reported cases of *S. iniae* infections. This organism is a catalase negative, facultatively anaerobic organism that produces a capsule and when plated onto blood agar demonstrates β-hemolytic activity.

In this study, the relationship between the hemolysin produced by *S. iniae* and the lactate oxidase gene (*lct*O) encoded by this organism was examined. Lactate oxidase is responsible for catalyzing the oxidation of L-lactate with molecular oxygen. Based on cloning studies, it had been suggested that this gene has a role in the hemolysis caused by *S. iniae* (unpublished observations). Two methods were chosen to determine whether the gene encoding lactate oxidase is associated with this hemolytic activity. The first method was a direct approach involving the inactivation of the *lct*O gene through homologous recombination with an inactive form of the gene. The second method entailed a more indirect approach using transposon mutagenesis. Electransformation experiments resulted in a protocol to transform *S. iniae* cells (not reported at start of these studies),

however, the functional relationship between the hemolysin of *S. iniae* and the *lct*O gene could not be determined.

Also included in this study was a preliminary characterization of the hemolysin expressed by *S. iniae*. This hemolysin is found extracellularly and begins to be produced at the beginning of the exponential phase of growth (strain ATCC 29178). It is believed to be a protein of greater than 12000 MW and demonstrates reduced activity when subjected to treatment with proteinase K suggestive of a protein moiety. The hemolytic activity also showed stability at elevated temperatures and incubation with cholesterol resulted in reduced hemolytic activity.

This thesis is dedicated to my husband Michael who was very patient throughout the writing of this paper and to my daughter Kasey Elizabeth who kept me endlessly entertained during the entire process.

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INTRODUCTION

Microorganisms can be found practically everywhere, including places that are unsuitable for higher forms of life. Most microorganisms however are found in the soil, water, on the surfaces of higher organisms and within plants and animals. The mammalian body contains billions of bacterial cells either found on or within the body. Microorganisms such as *Staphylococcus epidermidis* and various species of *Corynebacterium* are associated with healthy body tissues and are commonly referred to as the normal flora of the body. Other bacteria such as *Escherichia coli*, *Enterococcus faecalis,* and several species of *Bacteroides* are also considered part of the normal microflora and are found in the digestive tracts of humans (30,37).

The family Streptococcaecae is a large group of bacteria that play very important roles in everyday human life. This group of gram-positive bacteria is traditionally considered catalase negative, facultatively anaerobic cocci, which are usually found in pairs or chains. This family is normally divided into three genera: *Lactococcus, Enterococcus,* and *Streptococcus*. This diversified group of bacteria is beneficial to humans in a variety of ways including playing a role in primary host defenses, involvement in fermentative processes such as homolactic fermentation (37), and in other dairy processes (24). The bacteria involved in the primary host defense are very important. They play essential roles in the overall health of the person by limiting pathogen growth through competition of nutrients and provide a barrier against foreign bacterial invasion. The bacteria involved in primary host defenses can be found in almost

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bacterial invasion. The bacteria involved in primary host defenses can be found in almost every part of the body. Members of the genus *Streptococcus* are commonly found in three areas of the body. *S. sanguis, S. mutans* and various other streptococci can be found in the oral cavity, the gastrointestinal tract harbors *Enterococcus faecalis* in large numbers within the large intestines, and the respiratory tract contains many different streptococcus species including the potential pathogens *Streptococcus pyogenes* and *Streptococcus pneumoniae* (37). There are also many other species of streptococci, which play important roles in human metabolic processes such as the lactic acid producing bacterium *Streptococcus thermophilus*. This organism is involved in lactose digestion in the intestinal tract (24).

Streptococci are also associated with disease. These pathogenic bacteria can include those found in the normal flora such as *S. pyogenes*, which are found in the nasopharynx part of the upper respiratory tract of host carriers. This bacterium is responsible for causing some potentially life-threatening diseases such as scarlet fever, pharyngitis, septicemia, and in some cases fulminant systemic infections, which can lead to necrotizing fasciitis, with a 30% death rate (37). *S. pneumoniae* also found in the upper respiratory tract is responsible for life threatening infections such as bacterial pneumonia and meningitis (47). Another streptococcal pathogen, which is a particular threat to neonates, is *Streptococcus agalactiae.* This encapsulated organism gains access through aspiration of infected amniotic fluid and is responsible for neonatal pneumonia, sepsis and meningitis (42,50).

Streptococcus iniae is a streptococcal species associated with the ability to cause disease in fish as well as humans (14). This gram-positive bacterium is found associated

with both freshwater and saltwater fish (45). It is believed that *S. iniae* is also found in low numbers within the environment including both the surrounding water and deposits (43). *S. iniae* was first isolated in 1972 from an Amazon freshwater dolphin (*Inia geoffrensis*) (45). Since this initial report, there have been numerous cases of bacterial infection documenting involvement of different strains of *S. iniae*.

S. iniae is a non-sporulating, facultatively anaerobic bacterium that arranges itself into pairs or chains (19,43). These chains have been observed as long as ten cells in length (44). The size of the cocci was initially reported as 1.5 μM in diameter (45), however, variability in size has been observed (17). The cells demonstrate increased growth on brain-heart infusion agar, sheep's blood agar, and tryptose (43) and exhibit inhibited growth at pH 9.6, in NaCl solution of 6.5%, and 40% bile salts solution (44). This organism also possesses a capsule (45) composed of an uncharacterized matrix, which could play a role in the virulence expressed by this organism (47). When cultured onto sheep blood agar, *S. iniae* demonstrates β-hemolysis around the colony with a wider zone of α-hemolysis surrounding this inner zone of hemolysis (45). Other than this initial characterization on the hemolysin produced by this organism, data on the hemolysin produced by *S. iniae* has been limited.

Hemolysins are exotoxins that act by causing changes in the integrity of the hosts' cell membrane. There are two groups of hemolysins; the first group is the phospholipases. These toxins act by removing the charged head groups from phospholipids within the bilayer of its target cell. The second group of hemolysins is the pore-forming toxins, which form pores within cell membranes allowing for leakage of cell contents. Such activities may allow bacteria that have been taken up by phagocytes

to escape from vesicles by destruction of the surrounding membrane (47). There are a number of toxins produced by gram-positive bacteria including the hemolysins streptolysin O, streptolysin S, both of which are produced by *S. pyogenes*.

Thiol-activated hemolysins such as the pore-forming streptolysin O (SLO), and pneumolysin of *Streptococcus pneumoniae* can inhibit the respiratory burst of neutrophils, and stimulate production of the cytokines IL-1 and TNF, which results in an inflammatory response (41). SLO is an immunogenic toxin released during the exponential-phase of growth that is reversibly inactivated by oxidation, and inhibited by small amounts of cholesterol (10). The hemolysin SLS on the other hand is a non-immunogenic toxin that is unaffected by oxidation and demonstrates no inhibition when exposed to cholesterol. SLS activity has not been detected unless a carrier molecule is also present. The carrier molecule acts as an inducer of the hemolysin, resulting in activation of the toxin. RNA core and lipoteichoic acids are the most common and potent inducers. The SLS is associated with damage to erythrocytes, liposomes, protoplasts, and bacterial cells (36). Whether the hemolytic activity associated with *S. iniae* shows characteristics similar to SLS or SLO has not yet been investigated.

S. iniae possesses a gene (*lct*O) associated with lactate oxidase activity (24). There are at least two types of known flavin enzymes that are involved in the oxidation of L-lactate; one of which is the enzyme 2-monooxygenase, and the other lactate oxidase (15). The gene encoding lactate oxidase, known as *lct*O catalyzes the oxidation of the molecule L-lactate and utilizes molecular oxygen as the electron acceptor. The end products produced by this reaction are pyruvate and hydrogen peroxide (24). The fact that hydrogen peroxide is known to be detrimental to bacteria indicates that production of

this compound as an end product may benefit the organism in some way. The enzyme associated with lactate oxidase activity has been poorly studied and has only been examined in a few bacteria such as *Aerococcus viridans, Streptococcus faecalis,* and some species of *Pediococcus* (15).

S. iniae is a major cause of disease in fish and can cause a variety of symptoms in both freshwater as well as marine fish. The types of fish affected include tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhyncus mykiss*), yellowtail (*Seriola quinqueradiata*), Japanese flounder (*Paralichthys olivaceus*) and the common carp (*Cyprinus carpus*). Symptoms demonstrated by infected fish include those of bacterial meningoencephalitis, bilateral exophthalmia (pop-eye), clouding of the lens, poor appetite, erratic swimming, and death within 1-2 weeks (18). Internal symptoms can include enlargement of the kidney, liver, and spleen. Hemorrhages are also often seen in the anal zone of affected fish (44).

Every year fisheries lose fish by the tons due to this bacterium. The organism has been isolated in various locations such as Taiwan, Spain, South Africa, Japan and the U.S (18). In 1992, a Texas fish farm reported 75% mortality among one of their commercial fish, hybrid tilapia, as a result of *S. iniae* infection (44). In an Israel fishery, mortality rates as high as 50% in trout and 30% in tilapia have also been reported (18). It has been shown that the virulence of this organism differs with strain. In the study by Eldar et. al (18), highly virulent strains had an LD_{50} following cultures *in vitro* of 10^7-10^8 cfu, with an increase of virulence observed after three passages *in vivo* (LD_{50}: 10^2-10^3 cfu). The virulence of this organism seems to be associated with a specific genetic profile as well. Fuller et. al. (23) showed that both disease-associated and commensal strains were able to

adhere and invade human epithelial cells, however, only the disease-associated strains were able to resist phagocytic clearance and promote cellular injury to the host.

S. iniae responds to a number of different antibiotics including: tetracycline, vancomycin, erythromycin, and nitrofurantoin (45). However, by the time the organism is detected, it has usually infected large numbers of the fish population. The need for a vaccine has been acknowledged and the development is currently underway. One type of vaccine for this organism was prepared using whole-cell formalin-killed preparations from a virulent strain of *S. iniae*. Various doses of this vaccine were given to farmed rainbow trout, vaccination each fish twice. A month after the second dose was given, the fish were challenged with virulent strains of *S. iniae*. The fish were followed for a period of six months. It was found that the fish that had been vaccinated demonstrated a 90% protection response, while the controls showed 70-80% mortality rate. The vaccinated fish used in this study were able to withstand infection even upon second and third challenges with the bacterium for over four months under lab as well as field conditions (7,21).

Streptococcus iniae is also capable of causing disease in humans. The first human case of *S. iniae* disease was recorded in Texas in 1991. A second case was reported in Ottawa, Canada, in 1994. In the winter of 1995 through February of 1996 four new cases of disease due to this bacterium were reported in Ontario, Canada. In all these cases the disease was contracted through the handling of tilapia, a freshwater fish. Three of the patients recalled having an open wound at the time of handling the raw fish. The fourth patient had no recollection of having an injury prior to infection; however, this individual had been in bad health before the incident. The period of incubation ranged anywhere

from 16 hours to 2 days and symptoms included fever, pain swelling, cellulitis, skin infections, erythema, and meningitis (4). Upon administration of antibiotics (beta-lactams or clindamycin) all of the patient's symptoms subsided with no long lasting effects (4,5).

Due to the fact that this organism is becoming one of the prominent causes of disease in fish and has now been implicated as a possible human pathogen it is important to have a fast and effective way of identifying this bacterium. Two PCR methods that have been developed have proven to be fast and effective ways of identifying *Streptococcus iniae.* The first method involves the use of nested oligonucleotide PCR primers that are specific for the *S. iniae* 16S-23S ribosomal DNA intergenic spacer (8,20). The second identification method, also based on PCR, involves the use of the chaperonin 60 (Cpn60) gene identification method (26).

The purpose of this study was to partially characterize the hemolysin associated with *Streptococcus iniae* and attempt to ascertain whether the hemolysin gene is associated with the gene responsible for encoding the protein lactate oxidase. The reason for looking at this particular gene association is that when a DNA fragment encoding the 3 genes: *lct*O, *lct*P, and an ORF were cloned as a 4 kb fragment on a plasmid, the *Escherichia coli* host exhibited hemolytic activity. Further cloning and sequencing of each individual gene indicated the *lct*O gene as the gene most likely associated with this activity (unpublished observation). One method chosen to determine whether the *lct*O gene was associated with the hemolytic activity exhibited by *S. iniae* was *in vivo* recombination. By introducing, through electrotransformation, an inactive form of the *lct*O gene into the original *S. iniae* genome and assuming homologous recombination

occur, it could reveal whether the *lct*O gene was associated with the hemolytic activity by screening for loss of hemolysis.

Electrotransformation is a form of transformation in which a brief electrical pulse is applied across the cell membrane, which induces the formation of pores in the phosphlipid bilayer. This temporarily permeabilizes the cells allowing for the transport of exogenous DNA, antibodies, enzymes, and other biochemical reagents into the cell. The voltage used during an electrotransformation causes a rise in the transmembrane voltage to approximately 0.5-1.0 volts (52). The normal transmembrane voltage of a cell is approximately –.020 V to –.200 V, depending on the type of cell (1). This change in transmembrane voltage (membrane potential) increases the likelihood that DNA transport will take place through the open pores. Electrotransformation is used for a number of different processes including the transport of both large and small molecules into the cell and even in some cases, has involved the transport of particles such as viruses (52).

Before an electrotransformation can be considered as a means for introducing exogenous DNA into a cell, there are a number of factors that must be taken into account in order to obtain a successful transformation. One of the obstacles that could hinder the ability to transform a bacterium is its cell wall. The gram-positive wall generally presents more difficulty during electrotransformation than the gram-negative wall because the former contains more peptidoglycan layers. These layers makes up approximately 50% of the total cell wall thickness of gram-positive organisms in contrast to the 5-10% found in gram-negative organisms and gives them the advantage of being more resistant to environmental stress (48). The use of glycine and threonine are

common ways of weakening the cell wall without destroying it, thereby allowing a reversible pore to form. The size of the plasmid used may also affect transformation efficiency. In general, the smaller the plasmid used, the higher the transformation efficiency (40). Another factor influencing uptake is the stage of growth of the cell population when the electrotransformation takes place. It is generally believed that the optimal time to electroporate is when 20%-50% of cells lyse upon electroporation (16). An additional factor to be considered is whether there is another plasmid already present in the recipient organism. If another plasmid of the same incompatibility group were already present, this would result in the expulsion of the newly introduced plasmid from the cell (40). Cell stress can also result in an unsuccessful electrotransformation. This can occur due to the nonspecific chemical changes within the cell that occurs during the acquisition of exogenous DNA into the intracellular environment of the cell. Survival of the cells is dependent on the osmotic stability of both the extracellular and intracellular environment. It is important to consider the extracellular environment because when the molecular exchange occurs, this can lead to a chemical imbalance resulting in cell death if the stress on the cell is too great. All of these factors need to be considered and are dependent on the organism being used. Each time a new bacterium is used, it requires reevaluation of the optimal conditions (52).

In this study, electrotransformation was utilized for the introduction of an inactive form of the *lct*O gene into the bacterial cell in order to ascertain whether this gene was associated with the hemolysis demonstrated by *S. iniae*. Following transformation, the inactive form of the gene could undergo homologous recombination (34) with the original form of the gene within the bacterial genome, resulting in incorporation of an

inactive *lct*O gene. If the gene responsible for hemolysis is the same as the gene encoding lactate oxidase, then the organism would demonstrate loss of hemolytic activity. By identifying and characterizing the different factors responsible for or involved in disease processes of this organism, it would better enable veterinarians to treat infected fish much more efficiently and expeditiously and aid in further development of vaccines.

MATERIALS

Bacteria, Plasmids, and Growth Conditions

Streptococcus iniae strains used in this study are listed in Table 1. The *S. iniae* strain that corresponds to the wild-type strain ATCC 29178 was obtained from Dr. Alicia Gibello, Universidad, Complutense de Madrid. All other *S. iniae* strains were obtained from Jeff Fuller and Dr. Donald E. Low, Mount Sinai Hospital, Toronto, Canada. The *Escherichia coli* strains used as hosts for the plasmids included JM109 (Promega) and DBll (University of Oklahoma Health Sciences Center). Other bacterial species used were *Enterococcus faecalis* CG110 and *Lactococcus lactis* IL1837 obtained from the culture collection of professor J.J. Ferretti, University of Oklahoma Health Science Center. Plasmids used included pAT28 (51) and pIL253 (49).

S. iniae cultures were prepared by growing cells at 32°C in 5% CO_2 on Brain Heart Infusion agar plates. *E. coli* cultures were prepared by growing cells aerobically at 37°C with shaking at 220 rpm in 2X Yeast-Tryptone (2xYT) broth containing the appropriate antibiotic (250 μg/ml erythromycin or 100 μg/ml ampicillin). *E. faecalis* cultures were prepared by growing cells at 32°C in 5% CO_2 on Todd Hewitt agar plates containing 10 μg/ml tetracycline. *L. lactis* cultures were prepared by growing cells at 32°C in 5% CO_2 in Todd Hewitt broth.

TABLE 1. *Streptococcus iniae* strains used in this study

Strain	Source
ATCC 29178	Amazon freshwater dolphin
9034	Fish
9038	Fish
9103	Fish
9029	Human
9040	Human
9079	Human
9117	Human
9137	Human

Media

The following media were purchased from Difco laboratories, Detroit, MI and was prepared according to manufacturer's instructions: Todd Hewitt Broth (TH), Brain Heart Infusion (BHI), Mueller-Hinton Agar. Tryptone and Yeast Extract was purchased

from Fisher Scientific, Fair Lawn, NJ. Antibiotics were added when required as a sterile addition following preparation.

The preparation of solid media was by the addition of 1.5% (w/v) Agar (granulated) from Fisher Scientific, Fair Lawn, NJ to the liquid broth prior to autoclaving. The agar was poured into 100 X 15 mm polystyrene S/P diSPo petri dishes (Baxter Healthcare Corp., McGaw Park, IL). Upon solidification, plates were stored at 4°C. Sterile liquid media was stored in glass bottles at room temperature (RT).

Blood Agar (TSA with sheep blood) plates were obtained from Remel, Lenexa, KS. Plates were stored at 4°C prior to use. Fresh blood (sheep's blood / defibrinated) used for the hemolysin assay was obtained from Becton Dickinson and Company, Sparks, MD.

Chemicals

The following chemicals were obtained from Fisher Scientific, Fair Lawn, NJ: acrylamide (electrophoresis grade), bis-acrylamide (electrophoresis grade), ammonium sulfate, sodium acetate (enzyme grade), sucrose (ultra centrifugation grade), glycine (tissue culture grade), magnesium chloride (hexahydrate, enzyme grade), potassium chloride (enzyme grade), Tween 20 (enzyme grade), hydrochloric acid, sodium chloride (enzyme grade), Tris base (molecular biology grade), EDTA disodium salt (electrophoresis grade), glucose, sodium hydroxide (molecular biology grade), ethanol, potassium acetate (enzyme grade), isoamyl alcohol (biotechnology grade), chloroform (molecular biology grade), saturated phenol (pH 6.6/7.9), ammonium persulfate (electrophoresis grade), methanol, glacial acetic acid, brilliant blue R-250

(electrophoresis grade), TEMED (electrophoresis grade), agarose low EEO (electrophoresis grade), and ethidium bromide (electrophoresis grade).

The following chemicals and enzymes were obtained from Sigma Chemical Company, St. Louis, MO: RNase A, bromophenol blue, methylene blue, sodium dodecyl sulfate (SDS), cholesterol, ampicillin, spectinomycin (dihydrochloride), tetracycline (hydrochloride), mutanolysin, proteinase K, erythromycin, potassium tellurite, dithiothreitol 0.1 M (DTT) gelatin (type B: from bovine skin), Igepal CA-630.

Other enzymes and chemicals utilized in this experiment were from the following: Trypsin (bovine pancreas) was from Aldrich Chemical Company, Inc., Milwaukee, WI, lysozyme (hen egg white) was from Calbiochem, San Diego, CA. Hydrogen peroxide was from Kroger Family Pride, Cincinnati, OH.

Molecular weight markers used in this study were from the following: Lambda-Sty I DNA marker was purchased from MBI Fermentas, Amherst, NY, Mark 12 wide range protein standard was purchased from Novex, San Diego, CA; and Mid-Range protein molecular weight markers were purchased from Promega, Madison, WI.

Restriction enzymes and their respective reaction buffers were purchased from Life Technologies, Gibco BRL (Gaitherburg, MD), Promega (Madison, WI), and MBI Fermentas (Amherst, NY). These were utilized according to the manufacturer's instructions.

Dialysis membrane tubing with a molecular pore size of 12000 to 14000 MW was purchased from Spectral Por, Spectrum Company, Gardena, CA.

The Transporator plus electrotransformation system and the electrotransformation cuvettes (2 mm gap) were from BTX, Inc., San Diego, CA.

METHODS AND PROCEDURES

Initial Electrotransformation Protocol for *S. iniae*

Cloning and inactivation of the *lctO* gene. A plasmid containing an inactivated *lctO* gene was created by Dr. Wayne Hynes, Old Dominion University, Norfolk, VA. It was constructed from the *lctO* gene cloned into a pGEM-T Easy plasmid. The gene was digested using the enzyme MunI with the internal portion, approximately 369 bp in length replaced with an erythromycin resistance gene. The plasmid created containing the inserted erythromycin gene was given the name (for reference) pSIΔ*lctO*:erm (Figure 1A). Additionally, the internal *lctO* portion was cloned into the suicide vector p7erm, which contained an erythromycin gene on another part of the plasmid. This plasmid was given the name (for reference) pSIΔ*lctO*:munI (Figure 1B).

Plasmid mapping. Plasmid mapping using restriction enzyme digestion was used to determine the orientation of the *lctO* gene fragments and inserted erythromycin gene in the plasmid pSIΔ*lctO*:erm. Plasmid DNA was digested using the enzyme ScaI. This enzyme makes three cuts on this plasmid as shown in Figure 1. The first cut site is found on the vector portion of the plasmid, the second cut site is located 150 bp into the erythromycin resistance gene, and the third cut site is found 720 bp into the *lctO* gene. Digested DNA was run on a 0.8% TBE gel at 100 volts for 1.5 hours. Plasmid mapping was determined based on the size of the fragments. The plasmid pSIΔ*lctO*:munI was also digested using the enzymes EcoRI and HindIII. Each of these enzymes makes only one cut site on the vector portion of the plasmid and no cut sites in the internally cloned

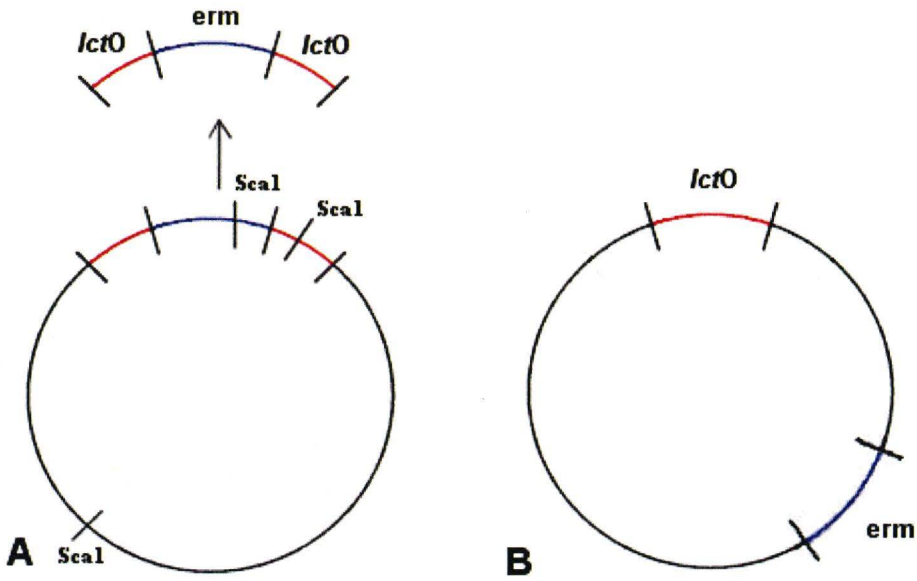

FIG. 1. Diagram showing the two plasmids used for specific inactivation of *lct*O. (A) pSIΔ*lct*O:erm fragment (referring to the inactive *lct*O gene fragment used for electrotransformation). Fragment was excised from the plasmid pGEM-T-Easy and used for electrotransformation. (B) pSIΔ*lct*O:munI (referring to the entire plasmid). The entire plasmid was used for electrotransformation.

*lct*O fragment. Digested DNA was again run on an 0.8% TBE gel at 100 volts for 1.5 hours.

Obtaining the *lct*O product for electrotranformation using PCR. Once it had been determined that pSIΔ*lct*O:erm contained the correct fragment length, the DNA primers PUC forward and reverse (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and (5'-CAGTATCGACAAAGGACACACT-3') were used to amplify this fragment for electrotransformation. These primers were obtained from IDT, Coralville, IA and were used at a concentration of 0.8 μM. PCR was performed using a Minicycler (PTC-150) instrument (MJ Research, Inc., Watertown, MA) in a final volume of 25 μL using 2 μL of diluted (1:50) DNA as template. The master mix was made as follows: 0.8μM of each primer, 1.6 μM dNTP mix, 1X of a 10X assay buffer B, 2.5 μM magnesium chloride, and 1.25 Units Taq DNA polymerase. Plasmid DNA templates containing the inactive *lct*O gene fragments were obtained from *E. coli* hosts using the Wizard Plus Maxiprep DNA Purification System (Promega). The PCR mixture was then subject to denaturing (94°C for 2 min) followed by 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 60°C for 1 min, elongation at 72°C for 2 min). A final elongation was performed at 72°C for 10 minutes. The fragment obtained was given the name (for reference) pSIΔ*lct*O:erm fragment.

Initial Electrotransformation Protocol. *S. iniae* electrocompetent cells were prepared by inoculating 50 ml BHI broth with 1 mL of an overnight culture (1:50 dilution) and grown at 32°C overnight. Bacterial pellets were collected (OD_{600} = 0.154 - .906 nm) by centrifugation using the Beckman J2-21 Centrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 7700 X *g* for 10 minutes at 4°C. Cells were washed with 2 mL

0.5 M sucrose solution and centrifuged at 12400 X *g* for 1 minute using the Eppendorf 5417c/R Centrifuge (Hamburg, Germany). The cells were then washed with 1 mL of 0.5 M sucrose solution and centrifuged at 12400 X *g* for 1 minute. This wash step was repeated a total of 5 times. After the last wash, pellets were resuspended in 50 µL of same solution and stored on ice until used. Electrotransformation of *S. iniae* was done using 50 µL electrocompetent cells with 5 µL of DNA (pSIΔ*lct*O:erm fragment or pSIΔ*lct*O:munI). Electrocompetent cells and DNA were combined in microcentrifuge tubes and transferred to 2 mm electroporation cuvettes. Samples were mixed thoroughly and electroporated at 2.5 kV as described in the manufacturers manual. Electrotransformed cells were then suspended in 1 mL TH broth and incubated at 32°C for a period of one hour. Cultures were concentrated by centrifugation and the cells were plated onto BHI agar plates containing erythromycin (2.0 µg/mL) and incubated overnight at 32°C in 5% CO_2.

Optimization of Electrotransformation Protocol for *S. iniae*

Bacterial strain determination. Bacterial colonies obtained after electrotransformation were preliminary ascertained to be *S. iniae* based on the following: colonies obtained on plates containing the antibiotic found on the transformed DNA were catalase negative, stained gram-positive, and were cocci occurring in pairs or chains.

Determination of optimal growth media in which to grow *S. iniae*. Bacterial cells were grown in various media including BHI, TH, and BHI supplemented with 0.50%, 0.60%, 1.0%, or 2.0% glycine. An overnight 5 mL culture was inoculated into 100 mL of media (1:20 dilution) and the cultures incubated at 32°C in 5% CO_2 for a period of 8 to 10 hours. One mL samples were removed every hour starting at hour 0 and the absorbance at 600nm recorded. A growth curve was determined using the above-mentioned media to establish at what time mid-logarithmic phase occurred. The data obtained from this analysis was graphed using Microsoft ®Excel program.

Determination of the presence of a plasmid in *S. iniae*. Several techniques were utilized to determine the presence of plasmids in different strains of *S. iniae*. The strains examined were ATCC 29178, 9137, 9079, 9029, 9040, 9038, and 9103. Three separate protocols were used to ascertain the presence of plasmid DNA in these strains.

The first protocol based on a collage of several different protocols used for isolation of plasmid DNA from various streptococcal strains was as follows: cultures were grown overnight in 10 mL BHI medium at 32° C. Cultures were centrifuged at 2350 X *g* for 10 minutes, the supernatant removed, and the cells washed twice using 5 mL 0.01 M Tris-HCl buffer (pH 7.5). After each wash, cells were centrifuged for 10

minutes at 4200 X *g*. Final cell pellets were suspended in 0.8 mL Tris-HCl buffer (pH 7.5) containing 4 M NaCl and mutanolysin (5 mg/mL). Cells were incubated in a 37°C water bath for 45 minutes. The cells were collected by centrifugation at 20800 X *g* for 5 minutes and resuspended in 400 µL lysis solution (1% SDS; 0.01 M Na_2-EDTA; 0.2 M NaOH) and put on ice 5 minutes. Three hundred fifty µL 3 M ammonium acetate (pH 5.0) was added, the suspension vortexed, and put on ice for a period of 60 minutes. Lysates were then centrifuged at 20800 X *g* for 10 minutes and supernatant collected in a fresh microcentrifuge tube (United Scientific Products). The supernatants were incubated at 95°C for 5 minutes and then put on ice for another 5 minutes. Five µL RNase A (5mg/mL) was added to each sample and incubated in a water bath at 37°C for 15 minutes. A phenol/chloroform extraction was performed using 400 µL of saturated phenol followed by an equal amount of chloroform. After each addition, samples were mixed by inversion and centrifuged at 20800 X *g* for 2.5 minutes. This extraction was performed twice.

A further 50 µL 3 M ammonium acetate (pH 5.0), and 1 mL cold 90% ethanol was added to each sample, and mixed by inversion before incubation at -70°C for 30 minutes. DNA was collected by centrifugation at 20800 X *g* for 12 minutes and the pellet air-dried. DNA was suspended in 25 µL TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA pH 8.0) and stored at 4°C.

The second protocol was modified from that described by Berridge, et. al (8), briefly, 10 mL samples were grown overnight in BHI broth at 32°C. Cultures were inoculated with 40 µL ampicillin (50 µg/mL) and incubated at 37°C for 2 hours. Ampicillin was used to weaken the cell wall of the organism. All samples were then

centrifuged at 13000 X *g* for 15 minutes. Cell pellets were resuspended in 1 mL lysis solution (50 mM glucose; 25 mM Tris, pH 8.0; 10 mM EDTA, pH 8.0; 150 mM NaCl) containing 100 μg RNAse (10 mg/mL), 50 μg mutanolysin (500 μg/mL), and 500 μg lysozyme (250 μg/mL). Cultures were then incubated at 37°C for an additional 1.5 hours. After incubation, 60 μL SDS (10%) and 50 μg/mL proteinase K were added and the samples incubated for an additional 1.5 hours at 43 °C. Phenol-chloroform (5:4) extractions were performed twice on each sample. The aqueous phase was collected and DNA precipitated using equal volumes of 90% ice-cold ethanol at -20°C overnight. The precipitated DNA was collected by centrifugation at 20800 X *g* for 4 minutes and DNA pellets air-dried. DNA was suspended in 25 μL TE buffer and stored at 4°C.

The third protocol used for the extraction of plasmid DNA was modified from the procedure described in Ausubel, et. al. (2). Cultures were inoculated into 6 mL BHI broth and incubated overnight at 32°C. One hundred μL of the overnight cultures were inoculated into 6 mL fresh BHI broth and incubated at 37°C for 6 hours. Cell pellets were collected by centrifugation at 13000 X *g* for 12 minutes, and resuspended in 1 mL GTE buffer (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA) containing lysozyme (25 mg/mL). Samples were incubated in a 37°C water bath for a period of 30 minutes after which 500 μL 0.2M NaOH/1% SDS was added to each sample and incubated on ice for 5 minutes for cell lysis to occur. After incubation, 350 μL of 5 M potassium acetate (pH 4.8) was added to the suspension and left on ice an additional 5 minutes. Samples were centrifuged at 20800 X *g* for 3 minutes and the supernatant collected. One-half volumes of phenol and $CHCl_3$:isoamyl alcohol (24:1) was added, mixed by inversion, and centrifuged at 20800 X *g* for 10 minutes. The aqueous layer was

collected into fresh tubes and the DNA was precipitated using two volumes of 90% ethanol. Samples were incubated overnight at -20°C. The precipitated DNA was collected by centrifugation at 20800 X *g*, washed twice using 70% ethanol, and air-dried. Pellets were resuspended in 25 µL TE containing RNAse (1 mg/mL).

Analysis of the presence of a plasmid. The plasmid extractions described above were analyzed electrophoretically using a 0.8 % agarose gel in TBE buffer (Tris; boric acid; EDTA) at 150 volts for one and one half hours. Gels were examined using a Gibco BRL UV transluminator (Life Technologies) with the photography by a Kodak DC120 Zoom Digital Camera (Eastman Kodak Co.).

The use of different *S. iniae* strains. Electrotransformation was performed as described below using various strains of *S. iniae*. Strains tested were ATCC 29178, 9029, and 9034.

Electrotransformation of *S. iniae* using two different plasmids. Two different plasmids were used to ascertain conditions suitable for the electrotransformation of *S. iniae*. These were pIL253 (51) and pAT28 (49). pIL253 (0.67 µg/µL) is a 4.8 kb plasmid that carries an erythromycin resistance gene, pAT28 (0.25 µg/µL) is a 6.7 kb plasmid that carries a spectinomycin resistance gene. Electrotransformations were performed as described below substituting either 3 or 5 µL of one or the other of these plasmids as the DNA.

Utilization of different electrotransformation wash solutions. Various electrotransformation wash solutions were used in the preparation of cells for electrotransformation. These solutions are listed in Table 2.

TABLE 2. Solutions used for the electrotransformation of *S. iniae*

Electrotransformation Wash Solution
1) 0.625 M sucrose, 1 mM $MgCl_2$, adjust to pH 4.0[a]
2) 15% Glycerol[b]
3) 0.5 M sucrose
4) 0.5 M sucrose using cells grown in BHI containing 0.6% glycine
5) 272 mM glucose, 1 mM $MgCl_2$, pH 4.0[b]
6) 7 mM potassium phosphate buffer, pH 7.4, 0.5 M sucrose, 1 mM $MgCl_2$[b]
7) 10 mM Tris-HCl, pH 6.0, 0.625 M sucrose/10 mM Tris-HCl, pH 5.0, 0.625 M sucrose/ 5 mM potassium phosphate buffer, pH 7.0/5 mM potassium phosphate buffer, pH 4.5, 1 mM $MgCl_2$, and 0.3 M raffinose[b]

[a] Obtained from reference (15).
[b] Obtained from reference (40).

Viability curve. A viability curve was generated to determine the voltage point at which 20%-50% of the cells are killed during electrotransformation. One hundred mL BHI broth was inoculated with 5 mL of overnight culture (1:20 dilution). Cells were collected by centrifugation (11100 X *g*; 15 minutes) and absorbance was recorded at 600 nm. Cells were electroporated as described below, however, different voltage levels (0.5

kV, 1.0 kV, 1.5 kV, 2.0 kV, and 2.5 kV) were used on each sample. After 1 hour incubation in 1 mL of BHI recovery broth, cells were diluted two-fold and 20 μL samples (X5) of each dilution were placed (drop-wise) on to the surface of BHI plates and incubated at 32°C in 5% CO_2 overnight. Colonies were counted on each plate to determine the number of colony forming units per plate. A control was also conducted using the same protocol except the cells were not subject to electrotransformation. The data obtained from this analysis was graphed using the Microsoft ® Excel program.

Optimal electrotransformation conditions. *S. iniae* electrocompetent cells were prepared by inoculating 100 ml BHI broth containing 0.6% glycine with 5 mL of an overnight culture (1:20 dilution) and growing at 32°C in 5% CO_2 . Bacterial pellets were collected by centrifugation using the Beckman J2-21 Centrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 12400 X *g* for 20 minutes. Cells were washed with 5 mL 0.5 M sucrose solution and centrifuged at 12400 X *g* for 20 minutes. The cells were then washed with 1 mL, 700 μL, 300 μL, and 100 μL of 0.5 M sucrose solution in succession. Samples were centrifuged at 20800 X *g* for 1 minute after each wash step. Pellets were resuspended in 80 μL of same solution and stored on ice until used.

Electrotransformation of *S. iniae* was done using 40 μL electrocompetent cells with 5 μL of plasmid DNA (0.27 – 0.61 μg/μL). Electrocompetent cells and plasmid DNA were combined in microcentrifuge tubes and transferred to 2 mm electroporation cuvettes. Samples were mixed thoroughly and electroporated at 2.5 kV as described in the manufacturers manual. Electrotransformed cells were suspended in 1 mL BHI broth and incubated at 32°C in 5% CO_2 for a period of three hours. Cultures were concentrated

by centrifugation and cells were plated onto BHI agar plates containing erythromycin (2.5 μg/mL) and incubated overnight at 32°C in 5% CO_2.

Extraction of plasmid DNA from electrotransformants. The protocol used for extraction of plasmid DNA from electrotransformants was a modification of the first protocol previously described that was used to determine the presence of plasmid DNA in *S. iniae*. The difference between these two protocols was that these samples were initially grown in BHI containing 20 mM D,L-threonine.

Attempted Inactivation of the *lct*O of *S. iniae* Using Electrotransformation

Electrotransformation utilizing the DNA pSIΔ*lct*O:erm fragment and pSIΔ*lct*O:munI. Electrotransformation of *S. iniae* using pSIΔ*lct*O:erm fragment or pSIΔ*lct*O:munI was performed using the optimal electrotransformation conditions determined previously. A diagram demonstrating the expected recombination events is shown in Figure 2. Figure 2A shows how the pSIΔ*lct*O:erm fragment would recombine within the original gene on the chromosome by a double homologous recombination event. In Figure 2B, pSIΔ*lct*O:munI would insert itself within the chromosome, disrupting the original *lctO* gene by a single homologous recombination event.

Detection of the inactive form of the *lct*O gene in transformants. To detect possible inactivation of *lct*O, a crude cell lysate procedure (28) followed by PCR was used on cells that showed erythromycin resistance. Cells isolated from electroporated cultures that demonstrated erythromycin resistance were grown overnight in 2 mL BHI broth containing 0.60% glycine. Cells were harvested by centrifugation (20800 X *g*) for

3 minutes and resuspended in 1 mL of 20% sucrose (w/v) in STE (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA, pH 8.0) containing lysozyme (25 mg/mL) and mutanolysin (50 U/mL). Cells were then incubated at 37°C for 1 hour, collected by centrifugation (20800 X *g*; 3 minutes) and resuspended in 1 mL lysis buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.3; 0.1 mg/mL gelatin; 0.45% Igepal CA-630; 0.45% Tween 20) containing proteinase K (100 μg/mL). Following incubation at 60°C for 1 hour they were heated to 95°C for 10 minutes. Two μL of each cell lysate was subject to PCR amplification. Primers used in this protocol were specific for the *lct*O fragment. The primers LCTO F (5'-TCACCTATAATTTGTAAGCG-3') and LCTO R (5'-CGACAAAGTTTTTAACAGAG-3') were obtained from Life Technologies, Inc., Gaithersburg, MD amplified the *lct*O gene.

Amplification with these primers amplify the *lctO* gene. PCR was performed using a final volume of 25 μL containing 0.8 μM of each primer, 1.6 μM dNTP mix, 1X of a 10X assay buffer B, 2.5 μM magnesium chloride, and 1.25 Units Taq DNA polymerase. The PCR mixture was subject to denaturation (94°C for 2 min) followed by 34 cycles of amplification (denaturation at 94°C for 1 min, annealing at 38°C for 1 min, elongation at 72°C for 3 min). A final elongation was performed at 72°C for 10 minutes. Amplified samples were assayed using five μL of PCR product to which 2 μL tracker dye was added. Samples were loaded on an 0.8 % agarose gel run at 150 volts for 1.5 hours. A molecular weight marker (lambda-StyI) was run on each gel to determine size of PCR products.

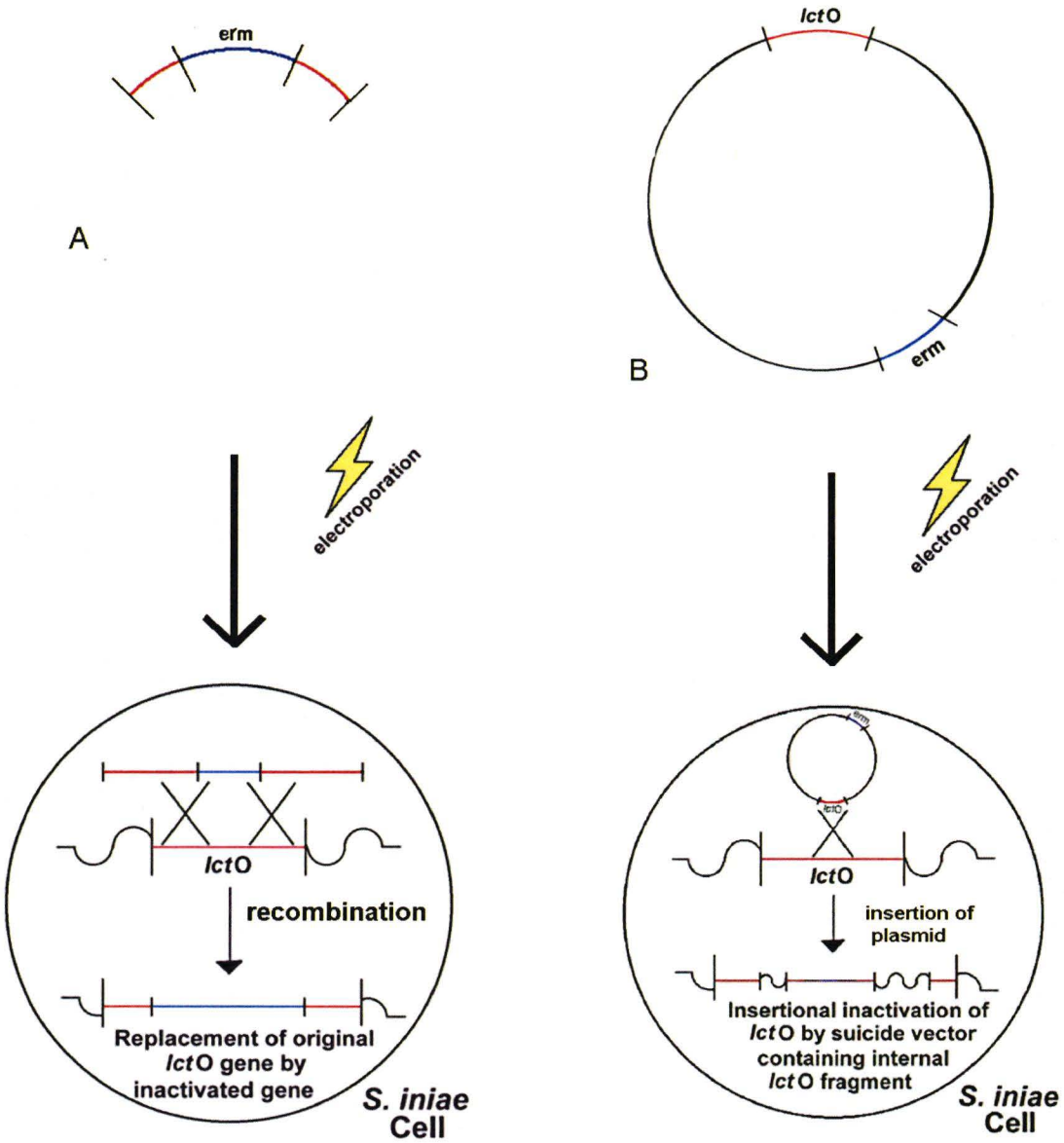

FIG. 2. Electrotransformation of the inactive *lctO* gene. (A) pSIΔ*lct*O:erm fragment replacing the original *lct*O gene by a double homologous recombination event. (B) pSIΔ*lct*O:munI disrupting the original *lctO* gene by a single homologous recombination event.

Preliminary Characterization of the Hemolysin of *S. iniae*

Preparation of red blood cells. Twenty mL whole defibrinated sheep's blood (Becton Dickinson Company, Sparks, MD) was centrifuged (J2-21 Centrifuge, Beckman Instruments, Inc., Palo Alto, CA) at 309 X *g* for 15 minutes. The top layer (plasma) was removed and 10 mL Hemolysin wash buffer (10 mM Tris pH 7.4; 160 mM NaCl) (25) was added, mixed gently by inversion and centrifuged another 15 minutes. This wash step was repeated until top layer appeared clear. A 2% RBC solution was obtained using 30 mL of hemolysin wash buffer with the addition of 0.67 mL washed RBC's. Whole blood as well as the 2% RBC solution was kept at 4°C. Red blood cell preparations were prepared fresh before an assay.

Hemolysin assay. Hemolysin assays were performed in 96-well microtiter plates (Fisher Scientific). Samples were assayed by preparing serial two-fold dilutions (1:2 to 1:1064) of each sample in hemolysin wash solution. Equal amounts of 2% RBC solution was added to each well and plates were incubated at 32°C in 5% CO_2 for a period of 2.5 hours. The supernatant was carefully removed and placed in a clean 96-well microtiter plate. Hemolytic activity was assessed by measuring the absorbance of each sample at 405 nm. The activity was recorded as either the absorbance reading or as the reciprocal of the highest dilution that exhibited at least a 70% reduction in hemolytic activity with respect to undiluted sample, and were obtained from the mean of two independent assays.

Isolation of the hemolysin from intracellular and extracellular cultures. Initial steps in isolating the hemolysin involved determining whether it was an intracellular or an extracellular compound. One hundred mL BHI broth was inoculated

from an overnight culture grown on blood agar. This was performed in duplicate. Cultures were grown overnight at 32°C in 5% CO_2. Following overnight incubation, the cultures were centrifuged at 12400 X *g* for 15 minutes. The supernatant was collected, filtered using a 0.45 μm filter and stored at 4°C. The pellet was resuspended in 1 mL hemolysin wash solution (pH 7.4), centrifuged (12400 X *g*; 15 min), and washed three times using 1 mL aliquots of hemolysin wash buffer and resuspended in a final volume of 1 mL. The cells were incubated at -20°C overnight. One mg of lysozyme was added to thawed samples, which were incubated in a 37°C water bath for 2 hours. Samples were then subject to lysis using a Mini-Bead Beater by Biospec products, Bartelsville, OK. Briefly, 1/3 volume of 0.1 mm diameter zirconia/silica beads were placed in microcentrifuge tubes along with cells. The cells/beads were then subject to shaking (5 X 1 min.) with 1-minute incubations on ice between each shaking. Samples were then put on ice for ten minutes, centrifuged (20,800 X *g*; 2 min), and the lysate collected and stored at -20°C.

To determine whether the hemolysin was found intracellularly or extracellularly both the filtered supernatant material and the intracellular lysate were tested for hemolytic activity. Briefly, 0.5 mL of each sample were pipetted into microcentrifuge tubes along with 0.5 mL 2% RBC, BHI was used as the blank. A control (100% lysis) of 0.5 mL water was run in parallel. Samples were incubated overnight at 32°C in 5% CO_2. Each sample was then centrifuged (420 X *g*; 20 min at 10°C). One hundred μL of the aqueous layer of each sample was pipetted into a 96-well microtiter plate and hemolysis was evaluated spectrophotometrically at 405 nm.

To concentrate the extracellular hemolysin, an overnight culture of 2.5 mL was

inoculated into 500 mL BHI (1:200 dilution) and grown overnight at 32°C in 5% CO_2. Overnight culture was centrifuged (12400 X *g*, 15 min at 10°C). Supernatant was collected and precipitated with ammonium sulfate (80 % saturation) by constant stirring at 4°C for 6 hours. Precipitated material was centrifuged (13800 X *g*; 20 min); the supernatant discarded and the pellet resuspended in 5 mL sterile cold water. After the pellet dissolved, the sample was dialyzed against hemolysin wash buffer for 3 hours; the hemolysin wash buffer was replaced with fresh buffer and dialyzed overnight at 4°C. Part of the sample was filtered through a 0.45μM filter, the other part of the sample was left unfiltered, both samples were stored at 4°C.

Determination of time of hemolysin production. To assess the time when *S. iniae* cells produce hemolytic activity a growth curve was performed, as well as assessment of hemolytic activity at every hour. Briefly, two 100 mL bottles of BHI were inoculated with 5 mL (1:20) of *S. iniae* cells ATCC 29178 and 9117 from overnight cultures. One mL of culture was removed at every hour for 10 hours with absorbance at 600 nm recorded. Samples were centrifuged (20800 X g; 2 min) and the supernatant collected. One hundred μL of each sample was pipetted into a microtiter plate. One hundred μL of 2% RBC solution was added to each well and plates were incubated at 32°C in 5% CO_2 for a period of 2.5 hours. After the incubation period, 50 μL of supernatant was removed from each well and the absorbance was recorded at 405 nm.

Freeze/Thaw extraction of hemolysin from solid media. This method was based on the protocol used by Johnson et al (31). Briefly, ATCC 29178 *S. iniae* cells were seeded onto a BHI plate and grown overnight at 32°C in 5% CO_2; following overnight incubation, plates were placed in -80°C freezer for 1 hour. A non-inoculated

BHI plate was incubated in parallel as a control. After the 1-hour incubation period, the plates were removed from the freezer and flipped over to collect the liquid in the plate lid as it eluted from the agar. Once the liquid had been collected, 200 μL of each sample was pipetted into a microtiter plate. Serial two-fold dilutions were performed (1:2 to 1:1064) in hemolysin wash solution. One hundred μL of 2% RBC solution was added to each well and plates were incubated at 32°C in 5% CO_2 for a period of 2.5 hours. After the incubation period, 50 μL of supernatant was removed from each well and placed into a clean microtiter plate. The absorbance was recorded at 405 nm.

Heat stability. Heat stability was determined by subjecting dialyzed unfiltered samples to different levels of heat. Briefly, 0.5 mL of sample was heated for 1 hour at the following temperatures: 37°C, 40°C, 50°C, 60°C, 70°C, 80°C, 95°C, and 100°C. After incubation at the various temperatures, 100 μL of each sample along with an equal amount of 2% RBC was pipetted into microtiter plates and incubated (32°C in 5% CO_2; 2.5 hours). After the incubation period, 50 μL of supernatant was removed from each well and placed into a clean microtiter plate. The absorbance was recorded at 405 nm. Determinations were made in duplicate.

Factors that influence/inhibit hemolysin activity. The effects of various enzymes and chemical agents on hemolytic activity were investigated by subjecting hemolysin samples to incubation in the presence of: lipase, cholesterol, trypsin, and proteinase K. All tested compounds (final concentration of 1mg/mL) were incubated (37°C; 1 hour) with dialyzed filtered sample. After incubation, samples containing lipase, trypsin, and proteinase K were boiled (100°C; 10 min) to inactivate the proteases. One hundred μL of each sample was pipetted into a microtiter plate, serial two-fold

dilutions were performed (1:2 to 1:1064) in hemolysin wash solution followed by the addition of 100 µL of 2% RBC solution to each well. Plates were incubated at 32°C in 5% CO_2 for a period of 2.5 hours. A control was run in parallel, also subject to serial two-fold dilutions, that was not subject to the various enzymes as well as the various agents used in this assay. After the incubation period, 50 µL of supernatant was removed from each well and placed into a clean microtiter plate. The absorbance was recorded at 405 nm.

PAGE (non-denaturing) gel/blood agar overlay method. Preparations were analyzed by electrophoresis under non-denaturing conditions by omitting SDS and 2-mercaptoethanol from 2X sample buffer. This protocol used the mini-vertical gel system (11). Using a 12.5% separating gel, and a 5% stacking gel. Fifteen µL of 2X sample buffer (2.5 mL 4X Tris-HCl pH 6.8; 2.0 mL glycerol; 0.1 mg bromphenol blue; and water to 10 mL) and 15 µL hemolysin sample were mixed and added to wells. Five µL Mark 12 Wide Range Protein Standard molecular weight markers were run on each gel. Thirty µL of 2X sample buffer was added to the alternating empty wells. The non-denaturing gel was run at 150 volts for 1 hour. After electrophoretic separation, half of the gel was laid (face up) over the top of a blood agar plate (36) and incubated overnight at 32°C in 5% CO_2. The second half of the gel was stained to determine if a protein band was present that would correspond to any hemolytic band found on the gel used on the blood agar plate. The gel was put into coomassie blue stain for a period of 1 hour with constant rocking. The stain was removed and the gel put into destaining solution consisting of 5% v/v methanol, 7% v/v acetic acid and 88% v/v water. The gel was kept de-staining overnight with constant rocking.

Inactivation of the Hemolysin Associated Genes Using Mutagenesis Analysis

Development of a *S. iniae* strain resistant to spectinomycin. *S. iniae* strains were needed that possessed resistance to an antibiotic different than that found in the bacterium *E. faecalis* in order to differentiate between these two bacterium. *S. iniae* (ATCC 29178) showed no resistance to spectinomycin. In order to obtain a resistant strain bacterial cultures were plated onto a series of BHI agar plates with increasing concentrations of spectinomycin beginning with 50 μg/ml. When resistance colonies were detected at one concentration, they were plated onto the next higher concentration. The concentrations were increased as follows: 100 μg/ml, 200 μg/ml, 250 μg/ml, 300 μg/ml, 400 μg/ml, and 500 μg/ml. Plates were incubated at 32°C in 5% CO_2 overnight.

Non-specific inactivation of the hemolysin gene using transposon 916. The protocol used for this part of the study was modified from that described by Hynes (27). Filters were prepared as followed: 0.20 μm metrical membrane filters (Gelman Sciences, Inc., Ann Arbor, MI) were submerged in 2X distilled water and then placed in filter apparatus (Swinnex –25 by Millipore). The lids were screwed on loosely and the filter apparatus, including the membrane filter, was wrapped in aluminum foil, leaving a vent in the top. Filters were then autoclaved for at 15 psi for 15 minutes at 121 °C on a liquid cycle.

Five mL BHI was inoculated with .05 mL *S. iniae* possessing resistance to spectinomycin up to 500 μg/mL and .05 ml *E. faecalis*/Tn 916 from overnight cultures. Samples were filtered through the filter apparatus and the cells collected on the membrane filter. The membrane filter was placed in the center of a BHI plate and

incubated overnight at 32°C in 5% CO_2. After overnight incubation, the membrane filter was removed from the plate, placed into 1 mL BHI and vortexed to resuspend the bacteria. One hundred μL aliquots were plated onto BHI/ 500 μg/ml Spec/10 μg/ml tet plates which were incubated overnight at 32°C in 5% CO_2.

Colonies that grew were sub-cultured onto fresh BHI/500 Spec/10 Tet plates and blood agar (in coordinating locations). Colonies that demonstrated loss of hemolytic activity were then subject to further testing.

Determination of strain possibly carrying transposon. Two assays were found that would selectively distinguish between *S. iniae* and *E. faecalis*. These assays would differentiate between these two strains based on the fact that *S. iniae* demonstrates inhibited growth when plated on 0.04% tellurite and 6.5% NaCl, whereas *E. faecalis* can grow on both types of media (12,33). Possible transposon containing strains were plated onto BHI agar plates containing 6.5% NaCl and BHI agar plates containing 0.04% potassium tellurite. These were incubated overnight at 32°C in 5% CO_2. Colonies were chosen that met the required criteria (no growth on either plate) for *S. iniae* cultures.

RESULTS

Determination of the Orientation of pSIΔ*lctO*:erm Using Plasmid Mapping

Plasmid mapping. Plasmid mapping using restriction enzyme digestion was performed to determine the orientation of the *lctO* gene fragments and inserted erythromycin gene in the plasmid pSIΔ*lctO*:erm. Figure 3 shows a plasmid map of pSIΔ*lctO*:erm, both possible orientations of the erythromycin gene, with relevant cut sites. Figure 4A shows the band sizes of 430, 2119, and 2395 bp in length produced from restriction enzyme digestion using ScaI. The size of these fragments indicates that the erythromycin resistance gene as well as the two *lctO* fragments flanking each side are oriented in a counterclockwise direction (Figure 4B).

Restriction enzyme digestions using the enzymes EcoRI and HindIII were also performed on plasmid pSIΔ*lctO*:munI to verify that the size of the inserted internal *lctO* gene fragment is the correct size. As shown in Figure 5A, the size of the DNA bands demonstrate fragment sizes 2568 and 313 bp in length which corresponds to the plasmid map seen in Figure 5B. The other DNA bands greater than 2690 bp are believed to be undigested DNA.

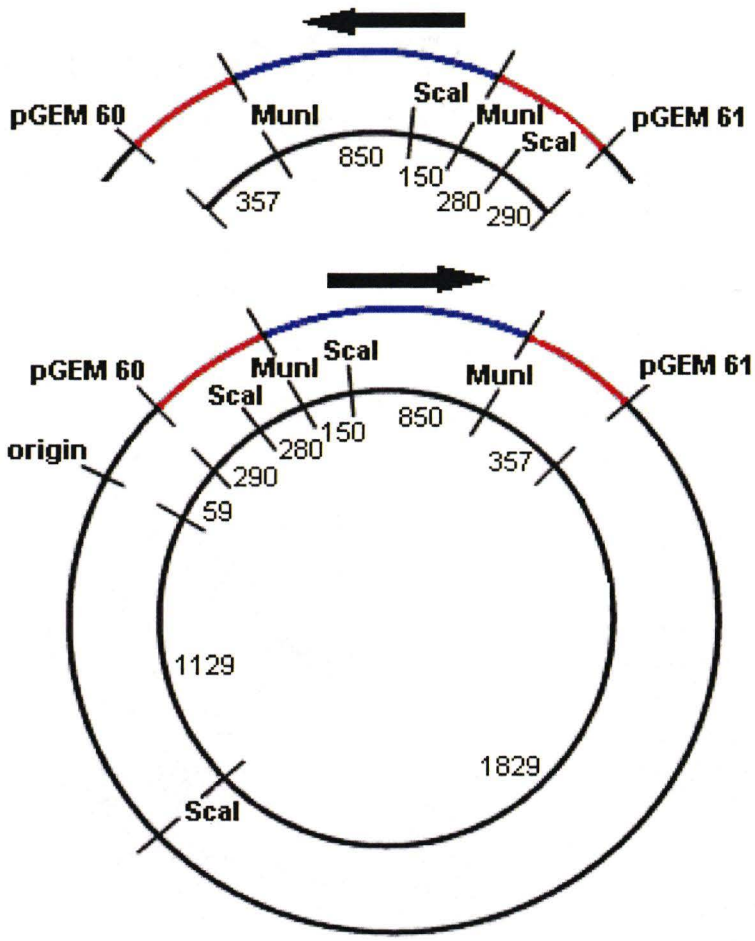

FIG. 3. Diagram showing a complete plasmid map of pSIΔ*lct*O:erm. *Lct*O fragments shown here in red, erythromycin resistance gene shown here in blue. The pGEM 60 and pGEM 61 indicated on the map are the sites on the pGEM plasmid where insertion of the pSIΔ*lct*O:erm took place.

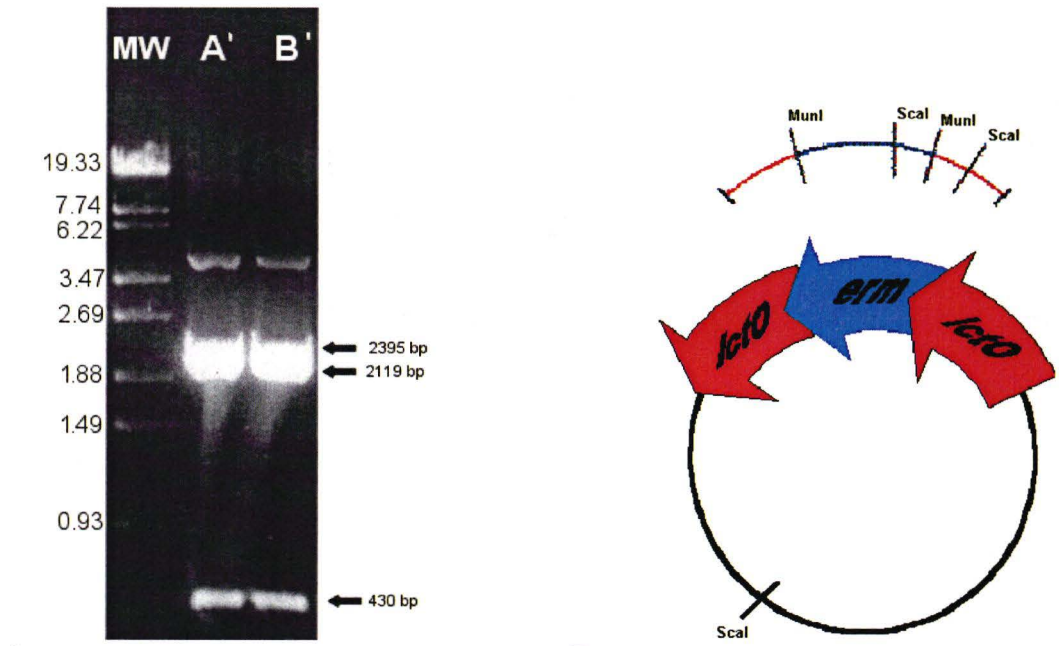

FIG. 4. Ethidium bromide gel and plasmid map demonstrating orientation of pSIΔ*lct*O:erm. (A) Gel showing fragments obtained from restriction enzyme digestions using Sca I. (B) plasmid map indicating orientation.

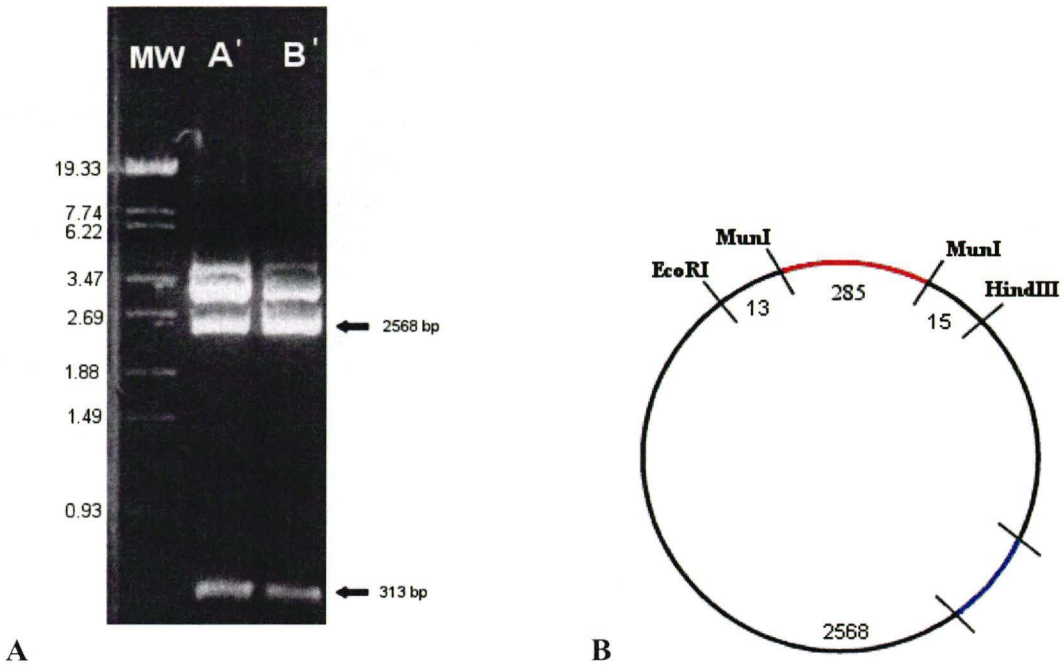

FIG. 5. Plasmid map and ethidium bromide gel demonstrating size of pSIΔ*lct*O:munI fragment (A) Plasmid map of pSIΔ*lct*O:munI. (B) Ethidium bromide gel showing fragments obtained from restriction enzyme digestion using HindIII and EcoRI. *Lct*O fragment shown here in red, erythromycin resistance gene shown here in blue.

Optimal Conditions for Electrotransformation

Electrotranformation conditions for *S. iniae*. In an electrotransformation there are many different variables to consider that can be altered in order to achieve the highest transformation efficiency possible. In the initial electrotransformation of *S. iniae*, a set protocol was used without variation to any of the parameters considered. The result of this electrotransformation was no transformants were obtained. Therefore, various parameters were varied to first determine the optimal conditions under which to transform exogenous DNA into competent *S. iniae* cells. A growth curve was first performed in both BHI and TH to determine the best broth in which to grow *S. iniae* cells. Figure 6 shows that this organism grows well in BHI broth, beginning with the exponential phase after 3 hours of growth and continuing through hour 10. The cells grown in TH also began the exponential phase at hour 3, however organisms in this broth showed a lower rate of growth throughout the entire growth cycle.

Cells were also grown in BHI containing various concentrations of glycine in order to determine the best concentration of glycine to use without interrupting the normal growth of *S. iniae*. As seen in Figure 7, all of the cells showed typical growth patterns in all of the concentrations except the medium containing 2% (266 mM) glycine. The cells showed decreased levels of growth as the concentration of glycine increased.

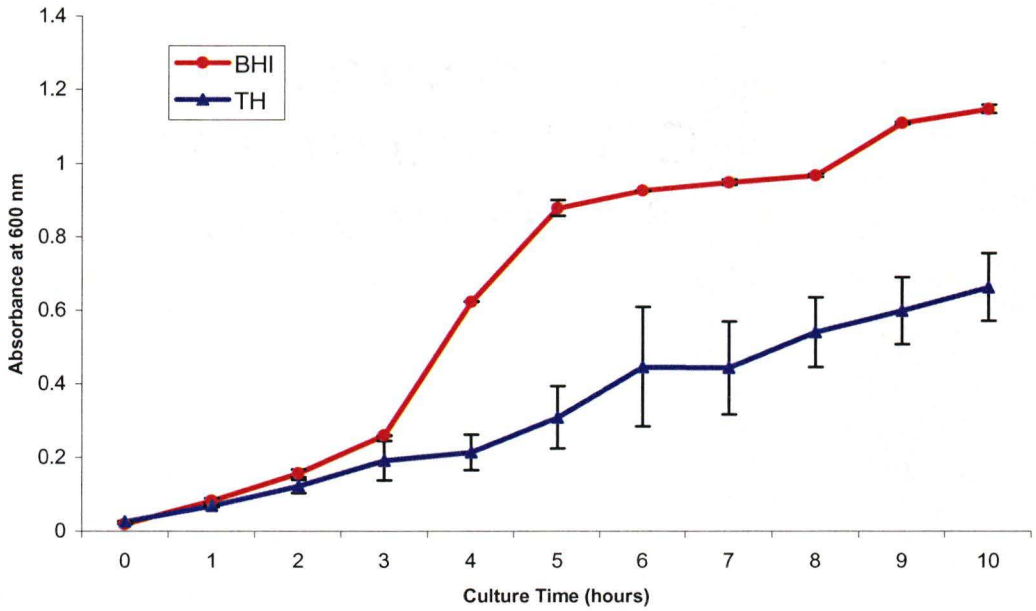

FIG. 6. Growth curve of *S. iniae* grown in Brain Heart Infusion broth and Todd Hewitt broth. Error bars demonstrate a variation in the growth of this organism in TH broth, whereas in BHI, *S. iniae* shows no variation in growth at all.

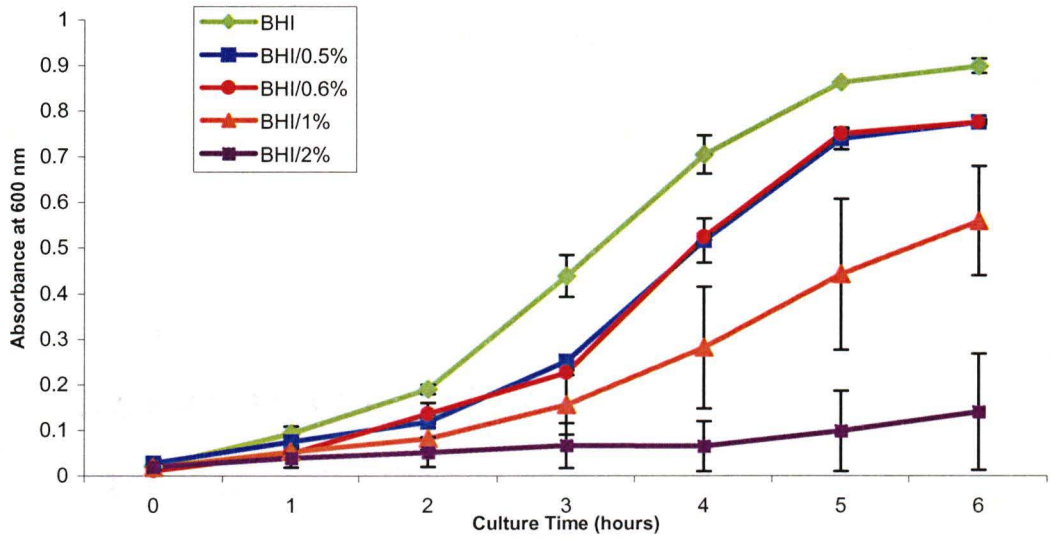

FIG. 7. Growth curve of *S. iniae* grown in BHI containing various concentrations of glycine. Error bars demonstrate the greatest amount of variation of growth in the BHI broth containing 1% and 2% glycine. There is an insignificant amount of variation seen in the growth of the organism in the other glycine containing broths tested.

S. iniae cells were assessed for the presence of another plasmid already present within the cell. Three different protocols were attempted to determine whether another plasmid was present within the cells. The presence of another plasmid can decrease the chances of a successful transformation (37). Gel electrophoresis was used to analyze the results of these extractions. Results suggested that the *S. iniae* strains tested do not contain a naturally occurring plasmid. After electrotransformation a plasmid was

extracted from transformed cells (see results at the end of this section) indicating that the plasmid extraction procedure works.

Other parameters can also be changed that could alter transformation efficiencies. These include the use of different sizes of plasmids, using different strains of *S. iniae*, different types of wash solutions, and the volume of DNA used. To look at the variability of plasmid uptake by *S. iniae* and also to determine the efficiency of uptake by different strains of this bacterium, the introduction of plasmids pIL253 (4.8 kb) and pAT28 (6.7 kb) was attempted using three different strains of *S. iniae* (ATCC 29178, strain 9029, and strain 9034). The resultant number of transformants varied. Table 3 shows that the pAT28 had a higher transformation efficiency in the ATCC 29178 strain (average of 10 transformants per μg DNA) than the pIL253, however, the pAT28 transformation reliability was unpredictable. Out of 6 electrotransformations using ATCC 29178, the pAT28 only yielded transformants in half of these experiments. Electrotransformation efficiency using pIL253 (average of 8 transformants per μg DNA) was slightly lower but demonstrated more consistency in producing transformants using the strain ATCC 29178. Plasmid pIL253 had better transformation efficiencies in both the *S. iniae* human (9029) isolate (average of 19 transformants per μg DNA) and the *S. iniae* fish (9034) isolate (average of 14 transformants per μg DNA) than the pAT28 plasmid, which gave no transformants.

TABLE 3. The number of transformants obtained after electrotransformation using different plasmids and different strains of *S. iniae*[a]

DNA[b]	Strain used	No. CFU	Avg. No. transformants/μg DNA
pIL253	ATCC 29178	10	8
pAT28	ATCC 29178	13	10
pIL253	9029	25	19
pAT28	9029	0	0
pIL253	9034	19	14
pAT28	9034	0	0

[a] All electrotransformations were performed using 0.625 M sucrose, 1 mM $MgCl_2$ (pH 4.0) as the electrotransformation wash solution. The volume of DNA used was 5 μL, and the voltage used was 2.5 kV.
[b] Concentration of DNA (pIL253, 0.27 μg DNA/μL and pAT28 0.25 μg DNA/μL).

Using varying concentrations of DNA during electrotransformation and varying the volume used can sometimes change the transformation efficiency as well. To examine these parameters, the plasmid pIL253 was used at a concentration of 1.54 μg DNA/μL in a 5 μL volume for electrotransformation. The number of transformants ranged from 1.0-87 transformants per μg DNA. When the volume of DNA used was reduced to 3 μL of the higher concentration (1.57 μg/μL) of pIL253, the number of transformants obtained was 8.0-177 transformants per μg DNA. A single plate

containing 137 transformants per μg DNA was obtained after an electrotransformation using 5 μL pIL253 at a concentration of 1.0 μg DNA/μL (Table 4).

TABLE 4. Number of transformants obtained after electrotransformation[a] using varying concentrations of DNA and varying the volume of DNA

DNA	Vol. Used	Cell Density[b]	No. CFU	No. Transformants/μg DNA
pIL253[c]	5 μL	0.622	740	137
pIL253	5 μL	0.508	150	19
pIL253	5 μL	0.673	652	85
pIL253	5 μL	0.777	673	87
pIL253	3 μL	0.673	815	177
pIL253	3 μL	0.836	37	8

[a] All electrotransformations were performed using 0.625 M sucrose, 1 mM $MgCl_2$ (pH 4.0) as the electrotransformation wash solution and the voltage used was 2.5 kV.
[b] Cell density was recorded as the absorbance reading in nanometers.
[c] The concentration of the DNA was 1.0 μg /μL, all other DNA concentrations were 1.54 μg/μL.

Changing the type of electrotransformation cell wash solution is another parameter that can be modified to change transformation efficiency. The type of wash solution used to wash the cells before transformation plays an important role in

transformation efficiency by changing the ionic strength of the bacterial suspension (22). In this study, 7 different wash solutions were utilized in electrotransformation using strain ATCC 29178 and the plasmid pIL253 as the exogenous DNA. Of these various wash solutions, the 15% glycerol, the 0.625 M sucrose/1 mM $MgCl_2$, the 0.5 M sucrose solution and 0.5 M sucrose solution using the cells grown in BHI containing 0.6% glycine were the only solutions that resulted in transformants after electrotransformation. Only 4 transformants per μg DNA were obtained from the 15% glycerol wash, whereas the 0.625 M sucrose/1 mM $MgCl_2$ had anywhere from 1 transformant per μg DNA to 17 transformants per μg DNA. The volumes of DNA were varied for the electrotransformations using the 0.5 M sucrose using the cells grown in BHI containing 0.6% glycine. The number of transformants obtained ranged from 35 transformants per μg DNA to 187 transformants per μg DNA (Table 5). Earlier electrotransformations were performed using 0.5 M sucrose wash solution, however colony counts were not recorded at that time.

TABLE 5. Number of transformants obtained by using different electrotransformation wash solutions[a]

Wash sol'n	Conc. DNA	Vol. DNA used	No. Transformants/μg DNA
1[b]	0.27 μg/μL	5 μL	2.0-17
2[c]	0.27 μg/μL	5 μL	4.0
3[d]	0.27 μg/μL	5 μL	No data available
4[e]	0.61 μg/μL	2.5 μL	187
	0.61 μg/μL	5 μL	134
	0.61 μg/μL	7.5 μL	127
	0.61 μg/μL	10 μL	35
5[f]	0.27 μg/μL	5 μL	0
6[g]	0.27 μg/μL	5 μL	0
7[h]	0.27 μg/μL	5 μL	0

[a] All electrotransformations were performed using *S. iniae* strain ATCC 29178 (original isolate), plasmid pIL253 and the voltage used was 2.5 kV. Note that the volumes and concentrations of the DNA varied for each wash solution used.
[b] 0.625 M sucrose, 1 mM $MgCl_2$, adjust to pH 4.0.
[c] 15% glycerol.
[d] 0.5 M sucrose.
[e] 0.5 M sucrose using cells grown in BHI containing 0.6% glycine.
[f] 272 mM glucose, 1 mM $MgCl_2$, pH 4.0.
[g] 7 mM potassium phosphate buffer, pH 7.4, 0.5 M sucrose, 1 mM $MgCl_2$.
[h] 10 mM Tris-HCl, pH 6.0, 0.625 M sucrose/10 mM Tris-HCl, pH 5.0, 0.625 M sucrose/ 5 mM potassium phosphate buffer, pH 7.0/5 mM potassium phosphate buffer, pH 4.5, 1 mM $MgCl_2$, and 0.3 M raffinose.

Another important parameter that alters the efficiency of a transformation is cell viability after electrotransformation. A viability curve is a graph that allows for determining when 20%-50% of the cells are killed during an electrotransformation procedure. As shown in Figure 8, it can be seen that when the cells are taken at an absorbance of 0.474, the optimal voltage to use is 2.0 kV. This is when there is a 20%-50% kill of the cells. When the cells are obtained at an absorbance of 0.622 the optimal voltage is 1.0 kV. The optimal time to perform the electrotransformation is dependent on the time at which the cells are collected, so in each instance it will be different.

By combining all of the different variables determined such as using a smaller size plasmid, growing cells in BHI containing 0.6% glycine, using 0.5 M sucrose electrotransformation wash solution, taking the cells at the correct time of growth and adjusting the volume of DNA and voltage used to increase efficiency at that time, an electrotransformation was performed that resulted in transformants (127 transformants/μg DNA) using the vector pIL253 (0.61 μg/μL). Plasmid was successfully extracted back out of the transformed *S. iniae* cells (Figure 9). Lane A shows a sample of the plasmid pIL253 used for the electrotransformation; lane B shows the extraction results of an extraction of a culture that exhibited antibiotic resistance after transformation but did not exhibit the presence of a plasmid upon extraction. The fact that the cell demonstrated antibiotic resistance but presented no plasmid suggests that the plasmid DNA could have recombined somewhere within the genome of the *S. iniae* cell or that the cell developed a mutation that provided it resistance to the antibiotic. Lane C is the result of an extraction from a transformed cell that showed antibiotic resistance after transformation, and resulted in the extraction of plasmid pIL253. These results demonstrate that the

parameters determined, such as the volume of plasmid, the electrotransformation wash solution, and the time and voltage at which to perform the transformation are effective in obtaining transformants.

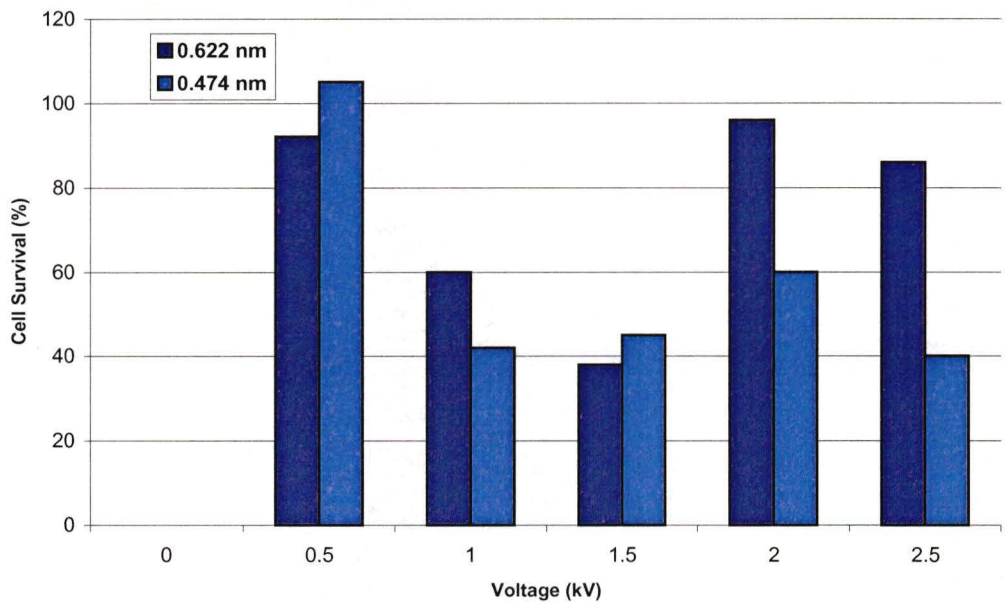

FIG. 8. Viability curve of *S. iniae* competent cells. Influence of transformation voltage on cell survival. Electrotransformation was performed (once per sample) using plasmid pIL253 in a 2mm cuvette at various voltages to determine when 20%-50% of the cells exhibited lysis. Cell densities were recorded as the absorbance reading at 600 nm. Viability curve was determined only once for each determined cellular density.

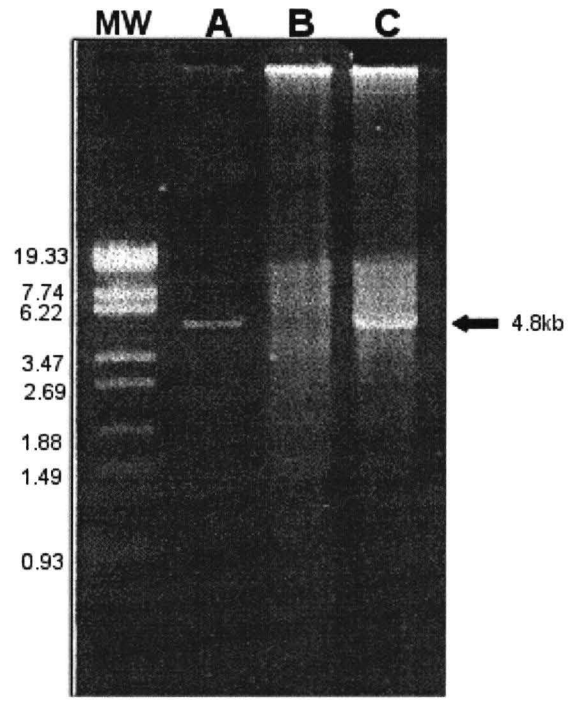

FIG. 9. Ethidium bromide gel demonstrating the extraction of plasmid pIL253 from transformed cells. (A) Plasmid pIL253 used as the exogenous DNA for electrotransformation. (B) DNA extracted from transformed cell that demonstrated antibiotic resistance but did not show the presence of a plasmid. (C) DNA extracted from resistant strain demonstrating the transformed cell possessed plasmid pIL253 after transformation.

Electrotransformation Utilizing pSIΔ*lct*O:erm Fragment

Using the parameters determined for successful electrotransformation of *S. iniae* cells, an electrotransformation to inactivate the *lct*O gene was attempted. The transformation of *S. iniae* cells with pSIΔ*lct*O:erm fragment (1.7 μg/μL) resulted in 23-26 transformants per μg DNA. Transformation using the pSIΔ*lct*O:munI only produced 2 colonies on the agar plate after electrotransformation. Transformants preliminarily identified as *S. iniae* cells based on morphology, gram stain, and catalase testing, were sub-cultured onto fresh BHI agar plates containing 2.5 μg/mL erythromycin. All colonies were also plated in parallel on blood agar plates to assay for hemolytic activity. Of the resultant colonies on the blood agar plates, only two transformants, presumably containing the pSIΔ*lct*O:erm fragment, demonstrated loss of hemolysis.

PCR was used to check for the inactive form of the *lct*O gene in the transformed cells. The primers used for detection of the inactivated *lct*O gene were the LCTO F and LCTO R as well as the pUC forward and reverse primers. The lctO primers amplified the band size expected for the wild-type *lct*O gene (Figure 10). All three samples show the same size band at approximately 1280 bp. A band corresponding to that expected for inactivation using pSIΔ*lct*O:erm fragment (1911 bp) was not detected. PCR amplification using pUC primers did not amplify anything.

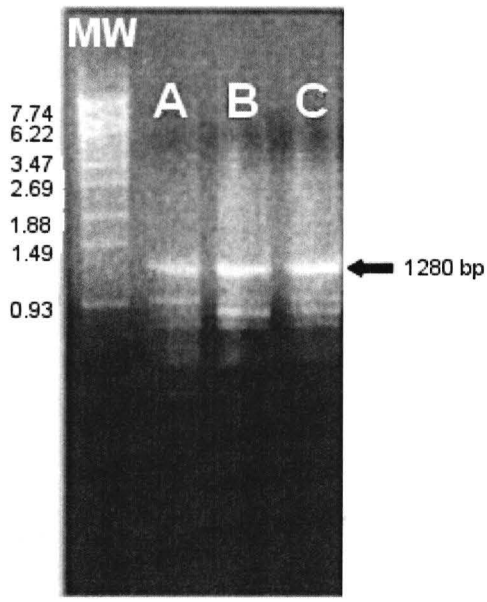

FIG. 10. Ethidium bromide gel demonstrating amplification of the original *lct*O gene using PCR in transformed cultures. Lanes A-C are 3 different transformants showing amplification of the original *lct*O gene within electrotransformed cells.

Hemolysin Characterization

Hemolysin characterization. The hemolysin from *S. iniae* strain ATCC 29178 was found in the extracellular matrix. The supernatant collected from the overnight cultures displayed higher hemolytic activity than the intracellular contents released by cell lysis (Table 6). The absorbance of the extracellular material was 4.56 times higher than the absorbance of the intracellular sample.

TABLE 6. Detection of hemolytic activity in intracellular and extracellular components[a]

	Absorbance[b]
Supernatant Collection	1.109
Intracellular Collection	0.243

[a] BHI was used as blank.
[b] Hemolytic activity based on absorbance reading (405 nm) following incubation with RBC.

A growth curve (performed in duplicate) on two different strains of *S. iniae* was done to determine when the hemolysin was produced. The strains chosen were the original dolphin isolate (ATCC 29178) as well as a human isolate (9117). The two strains had similar growth up through hour 3, at which time strain ATCC 29178 showed an increased growth rate through hour 6. The strain 9117 maintained a steady increase in growth throughout the entire growth cycle (Figure 11). Hemolytic activity was detectible

in the early stages of exponential growth for strain ATCC 29178 beginning at hour 5, whereas strain 9117 did not start showing any significant activity until hour 8. Both strains displayed a small amount of hemolytic activity at the beginning of the growth cycle, which was probably due to transfer of activity from the overnight culture. Throughout the entire growth cycle strain 9117 showed considerably less activity than the original strain ATCC 29178. Activity increased as the growth of the bacterial population increased.

Freeze/thaw extraction was performed to obtain the hemolytic activity from cultures grown on solid media. Supernatant obtained from liquid cultures did not demonstrate hemolytic activity when placed into wells cut in blood agar plates (Figure 12, well A), however, when incubated with red blood cells, the supernatant exhibited hemolytic activity. The liquid exuded from strain ATCC 29178 inoculated agar plates did show hemolytic activity as can be seen in Figure 12, well B, whereas, the liquid collected from a control plate (containing no inoculum) did not display this hemolytic activity (not shown). Freeze/thaw exudates collected from the plate inoculated with *S. iniae* ATCC 29178 also demonstrated hemolytic activity when incubated with RBC's, while the control plate had no hemolytic activity (Table 7).

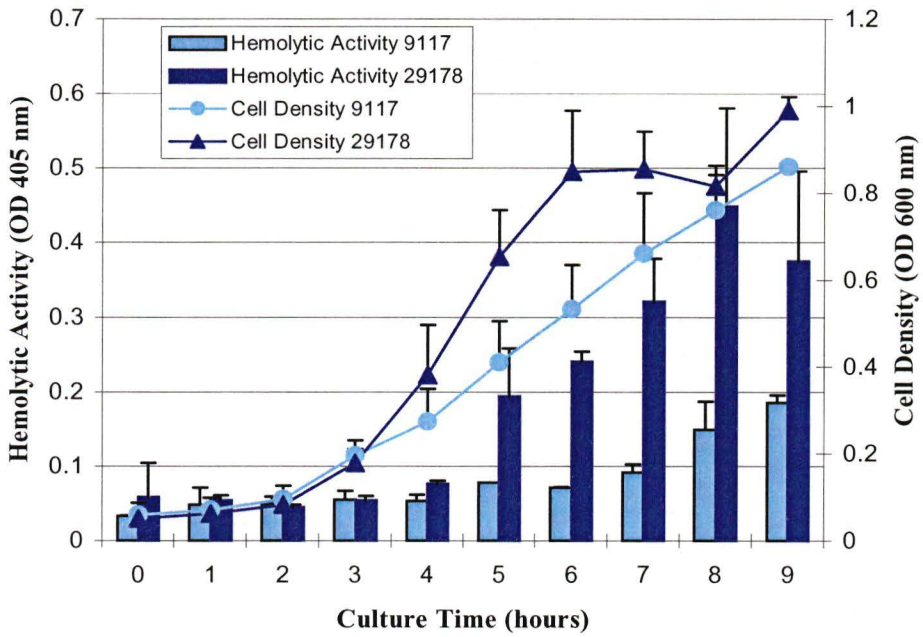

FIG. 11. Growth curve of *S. iniae* with time of hemolysin production. The growth curve was examined over a 9-hour culture time; cell density was based on an absorbance of 600 nm. The hemolytic activity observed was expressed as the absorbance reading of 405 nm following incubation with RBC.

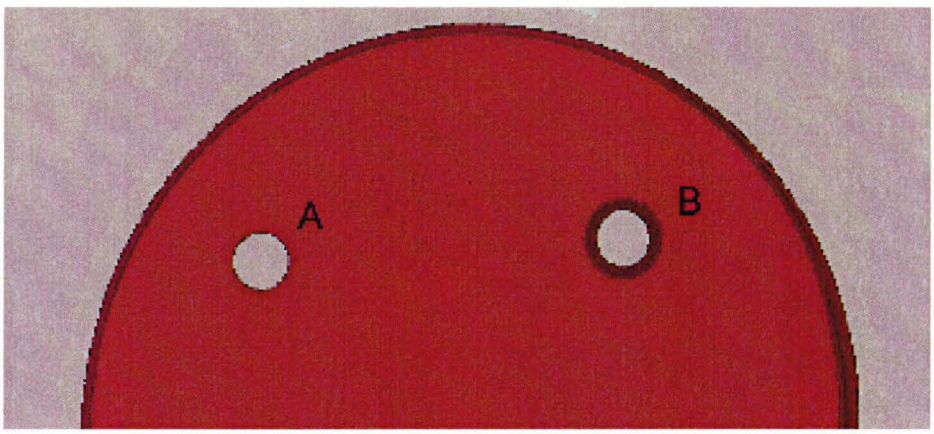

FIG. 12. Blood agar plate demonstrating hemolysis from freeze/thaw exudate. (A) supernatant collected from *S. iniae* overnight cultures grown in BHI broth. (B) exudate collected by freeze/thaw from plate containing *S. iniae* culture. Zone of clearing indicates hemolysis.

TABLE 7. Hemolytic activity detected from freeze/thaw exudates (strain ATCC 29178)

Freeze/Thaw Exudate	Initial Absorbance (nm)
Culture containing exudate	0.979
Control plate exudate	0.148

The heat stability of the hemolysin was assessed using temperatures ranging from 37°C to 100°C. This hemolysin showed a minor loss of activity when subject to temperatures ranging from 37°C to 95°C. As shown (Table 8), at 37°C, the hemolysin had lost 13% activity, at temperatures of 60°-95°C there was a slight increase in activity loss ranging from 24-27%. These results also show that the activity loss does not exhibit a steady pattern of loss in proportion with increase in temperature between the 60°-95° range. This is most likely due to the variance seen between the two absorbance readings used to obtain the average absorbance reading found in the table. When the hemolysin was subject to boiling for 30 minutes, it showed a 41% loss of activity as compared to the untreated material.

Hemolytic activity of the supernatant showed a 50% reduction of activity when incubated with the enzymes lipase and proteinase K and with the sterol cholesterol all resulting with a titre of 2 (Table 9). Hemolytic activity showed an increase when incubated with trypsin at a titre of 8. This is a two-fold increase in activity from the untreated material.

TABLE 8. Effects of heat treatments on hemolytic activity (strain ATCC 29178)[a]

Heat Treatment (%)	Absorbance (nm)	Std. Deviation	Loss of Activity
Untreated Material	0.970	+/- 0.007	
37°C	0.858	+/- 0.006	13%
40°C	0.811	+/- 0.015	18%
50°C	0.810	+/- 0.007	18%
60°C	0.743	+/- 0.004	25%
70°C	0.739	+/- 0.006	25%
80°C	0.717	+/- 0.018	27%
95°C	0.742	+/- 0.035	24%
100°C[b]	0.582	+/- 0.032	41%

[a] Samples were incubated for a period of 1 hour at 37°C and run in duplicate.
[b] Sample was subject to boiling for 30 minutes.

TABLE 9. Effect of various treatments on hemolytic activity (strain ATCC 29178)[a]

Treatment	HU[b]
Untreated Material	4
Lipase	2
Proteinase K	2
Cholesterol	2
Trypsin	8

[a] All samples were analyzed (in duplicate) at A_{405} nm.
[b] One HU is defined as the reciprocal of the highest dilution of the supernatant which caused at least 70% hemolysis.

A PAGE (non-denaturing) gel/blood agar overlay technique was used to separate the various proteins present in the supernatant and attempt to assess whether one of these bands corresponded with a hemolytic band produced by an analogous gel overlaid onto a blood agar plate. Figure 13 shows a zone of hemolysis from a gel overlay on the blood agar plate. However, this hemolysin band is unlikely the activity from *S. iniae*, but from the sample running buffer used in the experiment. When the gel was loaded with sample, a 2X sample running buffer (4X Tris-HCl pH6.8, glycerol, bromphenol blue, and water) was also loaded in between each well containing sample. Figure 13 shows a continuous hemolysis band the width of the gel, suggesting that the only zone of hemolysis seen is associated with the 2X sample running buffer. To further examine this effect, a gel was run containing only 2X sample running buffer. This gel revealed an identical hemolytic

run containing only 2X sample running buffer. This gel revealed an identical hemolytic band to the one containing alternating samples of hemolysin protein and 2X sample running buffer.

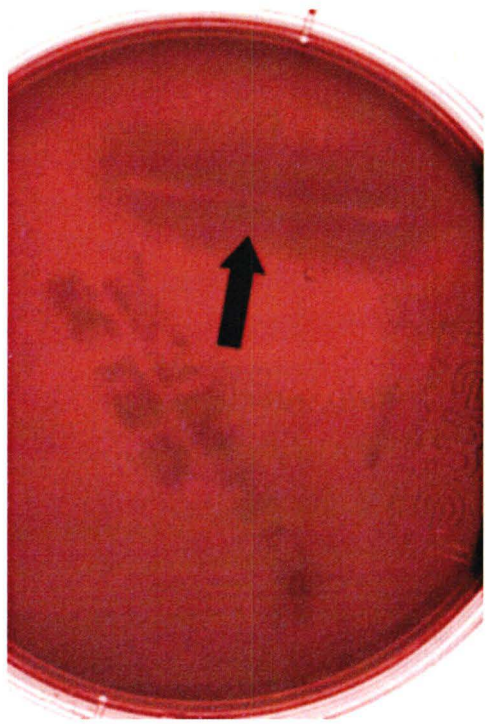

FIG. 13. Blood agar plate displaying hemolytic activity obtained from blood agar/overlay analysis.

Characterization of the Hemolysin Using Non-Specific Mutational Analysis

Mutational analysis. Nonspecific inactivation using transposon 916 was attempted to inactivate the *S. iniae lct*O gene. The use of transposons are considered nonspecific because the transposon has the ability to insert into DNA (chromosome and/or plasmid) at random positions (34,38).

The mating of *Enterococcus faecalis* donating cells carrying Tn916 with spectinomycin resistant *S. iniae* recipient cells resulted in production of *S. iniae* transconjugants with tetracycline and spectinomycin resistance. Upon subculturing onto blood agar plates some colonies demonstrated loss of hemolytic activity. These colonies were chosen to examine further based on this loss of activity. As further examination of the colonies were conducted, the colonies subsequently regained their ability to hemolyze red blood cells. Further experiments were performed to again obtain tetracycline and spectinomycin resistant *S. iniae* colonies demonstrating loss of hemolytic activity, however, these experiments using *S. iniae* cells carrying resistance to 500 μg/μL spectinomycin showed resistance to tetracycline as well, the antibiotic carried by the transposon.

DISCUSSION

Streptococcus iniae is a capsulated gram-positive bacterium responsible for diseases in freshwater and marine fish, as well as in humans (4). This organism has become a great concern in recent years among fisheries because of the high mortality rates it causes among fish and the constraint it could impose on fisheries (44). It has also gained much attention as an emerging human pathogen able to cause fulminant soft tissue infections (23) and meningitis (3). By examining the characteristics of this organism, a better understanding of *S. iniae* would be achieved that could aid in the prevention of future outbreaks among fisheries and humans as well. In this study, various properties of *S. iniae* were investigated. First, an attempt was made to establish a suitable protocol for the introduction of plasmid DNA into *S. iniae* by electrotransformation. This required determining suitable conditions including the best type of broth to grow the organism in, the type of electrotransformation wash buffer to use, the effects of using different plasmids, which strain of *S. iniae* to use, the optimal time during the growth period to use *S. iniae* cells for electrotransformation, and determination of whether naturally occurring plasmids could interfere with electrotransformation.

It was determined that this organism grows better in BHI broth than in Todd Hewitt broth and follows a typical growth pattern in BHI containing glycine at a concentration of 1% or less. Glycine or other agents are sometimes included in protocols for cells used in procedures such as electrotransformation to weaken the cell wall. Bacterial organisms especially gram-positive organisms such as *Streptococcus salivarius* and *Streptococcus sobrinus* (13) are very resistant to physical stress due to the thick outer

wall they possess. Therefore, it is useful to add an agent, such as glycine or possibly cell wall hydrolyzing enzymes, to aid in the weakening of the cell wall. By weakening the cell wall, the exogenous DNA is able to penetrate this barrier more easily (48). The problem with cell wall hydrolyzing enzymes in gram-positive species is that these organisms vary in their susceptibility to these enzymes. Glycine causes weakening of the wall by replacing both the D and L-alanines; which make up the interpeptide bridges in the pepetidoglycan layer of the cell wall (13). Using glycine is an efficient, inexpensive means of weakening the cell wall, without completely degrading the wall, in the preparation of cells for electrotransformation (48). However, determining the maximum concentration of glycine in which to grow the organism is important because too much glycine in the medium inhibits cellular growth and can result in cell death (16). From these results it was found that *S. iniae* cells grown in BHI containing concentrations of glycine at 1% or less are viable and would be suitable to use for electrotransformation assays. In this experiment, a concentration of 0.6% was chosen because it had less effect on growth than the 1% glycine concentration.

Another important parameter to consider when performing electrotransformation is the type of wash solution used in cell preparation. It is important to use a solution that lowers the ionic strength of the bacterial culture media before the transformation takes place (22). Too much stress on the cells due to changes in the ionic strength during electrotransformation can lead to cell death (52). It was shown that three wash solutions resulted in the transformants after electrotransformation. Results show that of these three solutions, 15% glycerol, 0.625 M sucrose/1 mM MgCl2, and 0.5 M sucrose solution using cells grown in BHI containing 0.6% glycine, the later two solutions gave much

higher transformation efficiencies than the other solution. Also noted here is the fact that the volume and concentration of the DNA used was different for the 0.5 M sucrose electrotransformation wash solution using cells grown in BHI containing 0.6% glycine. Due to this variance between the concentration and volume of DNA used with this wash solution and the other wash solutions, it is difficult to determine whether this particular electrotransformation wash solution was really one of the most efficient wash solutions or whether it was due to the higher concentration and volume of DNA used during the electrotransformation.

It has been noted in various experiments that the size of the exogenous DNA to be taken up during electrotransformation plays an important role in the success of the experiment. Generally, the smaller the plasmid, the higher the transformation efficiency (16). The transformation efficiency of *S. iniae* was assessed using two plasmids that vary slightly in size, pIL253 (4.8 kb) and pAT28 (6.7 kb). The pIL253 carried an erythromycin resistance marker, whereas pAT28 carried a spectinomycin resistance marker. Results showed that the *S. iniae* ATCC 29178 cells were able to take up both plasmids during electrotranformation, however pAT28 was not consistently taken up. The fact that both of these plasmids were able to become transformed into *S. iniae* ATCC 29178 cells is not surprising as the difference in plasmid size is only 1.9 kb. This is considered a relatively small difference in DNA size compared to the size differences found in other reports that base their findings of smaller plasmids being taken up more efficiently than larger plasmids (16,40). The largest plasmid reportedly used for electrotransformation was greater than 30 kb in size compared to the 5-7 kb plasmids that are normally used and have much higher transformation efficiency.

There is also variability in electrotransformation efficiency not only among organisms of the same genus, but among strains of the same species as well. It has been found that some strains of a certain species take up exogenous DNA more efficiently than others (13). A strain of *S. iniae* from the original dolphin isolate, a human isolate, and a fish isolate were assessed to determine whether one took up exogenous DNA better than another. When comparing the uptake of the two plasmids using these three strains of *S. iniae,* only pIL253 was taken up by all three strains. Transformants were never obtained using the plasmid pAT28. Overall, the three strains tested were able to take up the pIL253 plasmid with the same efficiency.

Varying the volume and concentration of DNA used during electrotransformation also had an effect on the transformation efficiency. When 3-5 μL volumes of DNA were added to the competent cells, higher numbers of transformed cells resulted after the experiment. However, a limit was observed, which, if surpassed resulted in no transformants. Addition of 10 μL DNA to the competent cells, resulted in arcing within the electroporation cuvette. This increase in conductivity across the cellular membrane was most likely due to the larger volume of DNA added to the cell suspension. The addition of DNA increases the salt concentration of the suspension, which also causes an increase in the conductivity of the cell, therefore, higher concentrations of salts found in the sample result in an increase in conductivity (52). Increasing the concentration of DNA also had an effect on transformation efficiency. It was found that higher numbers of transformants were obtained from higher concentrations of DNA, but this was dependent also on the volume of DNA added. Higher concentrations of DNA were

required in less volumes. Transformation efficiency decreased when larger volumes of concentrated DNA were used.

It has been shown in previous electrotransformation experiments that optimal transformation was achieved when 20%-50% of the cells lyse upon transformation (16). A cell viability curve was used to determine at what stage of growth there was 20%-50% cell lysis. This is not only based on the growth stage of the organism, but also on the voltage used during the electrotransformation. Based on the lysis curves obtained, it was found that when cells were taken at approximately mid-exponential phase, the optimal voltage to employ was 2.0 kV. This is when there is approximately a 20%-50% kill of the cells. When the cells are obtained at an absorbance of 0.622 (late mid-log phase) the optimal voltage is 1.0 kV.

Bacteria also possess certain characteristics that could hinder the efficiency of transformation. The presence of another plasmid of the same compatibility group within the cell can result in the expulsion of any newly obtained DNA when replication occurs (37). Not all plasmids are able to coexist in the same cell. Plasmids are divided into compatibility groups based on their origins of replication. When a new plasmid is introduced into a cell already containing a plasmid of the same compatibility group, the cell stops further plasmid DNA replication until the two plasmids have been segregated into different cells (34). Three different protocols were used to determine whether *S. iniae* cells contained a plasmid. No plasmids were detected in any of the strains tested by any of the methods used.

Electrotransformation is a process in which many different variables must be considered to achieve a successful transformation. The combination of all of these

different variables including growing the cells in BHI broth containing 0.6% glycine to weaken the cell wall, the use of 0.5 M sucrose solution as the electrotransformation wash solution, determining that at mid-log phase the optimal voltage to use was approximately 2.0 kV, assessing the difference between using one plasmid versus another, and using exogenous DNA in the volume of 5 μL resulted in a successful transformation. The plasmid pIL253 was transformed into *S. iniae* cells and then successfully extracted back out of the cell using the same procedures as was performed to try to assess whether *S. iniae* possessed a naturally occurring plasmid (Figure 4). The efficiency of this transformation was very low however. The transformation efficiency was 127-187 transformants per μg DNA. Generally, a good transformation efficiency based on various electrotransformation procedures is anywhere from 1 X 10^4 to 10^5 transformants per μg DNA (13, 16).

Once the variables were determined that resulted in electrotransformation these variables described in the above paragraph were used in procedures aimed at creating a mutant containing an inactive form of the *lct*O gene. The possibility of cellular growth inhibition can always exist when inactivating a gene whose function is uncertain. The function of the *lct*O gene of *S. iniae* is thought to be a possible detoxification mechanism, or a mechanism used for the assimilation of lactate as a source of energy when glucose is absent. A study by Gibello et. al (24) found that repression of the lactate oxidase enzyme of *S. iniae* was seen when the organism was grown in the presence of glucose and cellular growth inhibition was seen when exposed to high concentrations of lactate. These findings suggest that inactivation of the *lct*O gene could present a problem with

cellular growth if the organism is grown in the presence of excess amounts of lactate in the medium.

Electrotransformation to obtain colonies containing the inactive form of the gene resulted in colonies that had properties suggesting they were *S. iniae* and contained erythromycin resistance carried by the pSIΔ*lct*O:erm fragment since they were selected by growth on BHI/erm plates. To ascertain whether the interrupted form of the *lct*O gene had recombined with the chromosomal *lct*O gene, PCR amplification was utilized. Results indicated no recombination within the original *lct*O gene as no clones showed the inactive form of the gene. Figure 8 showed that the size of the amplified band was approximately 1280 bp, whereas if the inactive form of the gene had been amplified, the band would have been 1911 bp. These results suggest that the *lct*O gene was not inactivated.

The results obtained are perplexing because the bacterial cell exhibited erythromycin resistance, however, the inactive form of the gene did not recombine with the original *lct*O gene. These results strongly suggest that the inactivated form of the gene or some other portion of the pSIΔ*lct*O:erm fragment had enough homology with another region of the *S. iniae* genome to recombine at another location. If this occurred, the primers chosen to detect the natural *lct*O gene would not detect the inactive form of the gene at another location within the genome. The LCTO primers used in this study flank the *lct*O gene and are located approximately 30 bp outside of the gene on both the 5' and 3' end as determined using the program EditSeq ™ Expert Sequence Analysis Software. Therefore, these primers should not recognize other DNA sequences except those flanking the natural *lct*O gene.

Because the entire genome of *S. iniae* is unknown at this time, it is difficult to determine where this recombination would have taken place within the genome. A blast search was done using the known *lct*O gene sequence to determine if there are other genes with enough homology to this gene that would possibly result in recombination. The blast search using NCBI GenBank (http://www.ncbi.nlm.nih.gov/) revealed significant alignments with several genes including the *Mtf*1 gene found in *Saccharomyces douglassii,* and the lactate oxidase gene from *Aerococcus viridans*. The sequence homology between the *Mtf*1 gene and the *lct*O gene of *S. iniae* is interesting. The product of the *Mtf*1 gene (MTF1), a subunit of the mitochondrial RNA polymerase in yeast is involved in promoter recognition and has similarity to protein sequences of several members of the bacterial sigma factor family (39). The results of these findings suggests that *S. iniae* could possess a gene, possibly a sigma factor that is similar to the *Mtf*1 gene possessed by *S. douglassii* or another undiscovered gene at this time that would have enough similarity with the *lct*O gene fragment to result in recombination.

A second component to this study involved the use of transposons to determine any relationship between the *lct*O gene and the hemolytic activity of *S. iniae*. Transposons are transposable elements that have the ability to "jump" from one location in a chromosome to another (34) . Transposition can be within the same organism or it can involve transfer of the transposon to a different organism. Transposons are made up of two parts: a stretch of inverted terminal repeats, known as the insertion sequence (IS) region which allows the transposon to insert into the host genome and a piece of DNA that carries genes encoding specific phenotypes including the transposase which is needed for transposition (37). Transposons occur naturally and are found in both gram-

positive and gram-negative organisms. They carry genes for antibiotic resistance as well as encoding various enzymes such as transposase inhibitors and β-galactosidase (32). In this study the tetracycline resistant transposon 916, originally found in *E. faecalis* (32), was utilized in attempts to identify gene or genes associated with hemolysis. It was anticipated that the transposon, through random insertion would incorporate itself into a gene associated with this phenotype.

After mutagenesis some of the *S. iniae* cells exhibited loss of hemolytic activity, suggesting that the transposon had inserted into the gene associated with hemolysis. Using two different tests, it was determined that the cells demonstrating loss of hemolysis were *S. iniae* cells and not the donor cells *Enterococcus faecalis*. However upon subsequent cultures of these same cells onto blood agar plates, the *S. iniae* cells had regained their hemolytic phenotype. This probably resulted from excision of the transposon from its insertion site. It has been found that transposons, under certain conditions, have the ability to excise themselves from the genome (32). This instability is controlled by both the transposon as well as the recipient cell, therefore the success of mutagenesis studies is dependent on both components. Due to the observation that the organism initially demonstrated loss of hemolytic activity and then subsequently regained this phenotype upon subculturing, it is believed the transposon had excised itself out of the gene associated with hemolytic activity.

An additional problem that occurred was the development of resistance to tetracycline by *S. iniae* cells when made spectinomycin resistant, thereby making it impossible to differentiate it from the *Enterococcus faecalis* cells. The reason for this acquired resistance may have been because the antibiotics tetracycline and spectinomycin

have similar targets of action. Tetracycline inhibits bacterial growth by binding the 30S subunit of the bacterial ribosome. This results in distortion of the A-site of the ribosome, thereby preventing the tRNA from binding and interacting with the correct codon mRNA. Aminoglycosides such as spectinomycin also bind the 30S subunit of the bacterial ribosome. This results in the inability of the 50S ribosomal subunit to bind and subsequently inhibits protein synthesis (47). Since both of these antibiotics initially target the 30S ribosomal subunit, it is possible that the *S. iniae* cells already carrying the spectinomycin resistance gene acquired resistance to the action of the tetracycline as well. In a previous study, it was found that organisms already possessing resistance to the aminoglycoside streptomycin developed resistance to tetracycline upon continued exposure to this antibiotic which has a similar mechanism of action to aminoglycoside antibiotics (6). Future studies could involve the use of different donor to recipient ratios as well as using different transposons, which would possess an antibiotic resistance marker with a different mechanism of action than the spectinomycin resistant strains of *S. iniae* used in this study. Due to these circumstances, non-specific inactivation using transposons was discontinued.

In the present study partial purification of the hemolysin expressed by *S. iniae* was also attempted. The agent associated with hemolytic activity was isolated from the supernatant of bacterial cultures indicating it is synthesized and excreted into the extracellular matrix. Comparison of the growth patterns between the original *S. iniae* strain ATCC 29178 and human isolate 9117 in broth revealed early activity levels in strain ATCC 29178 beginning at hour 5, whereas in strain 9117, activity was not significant until hour 8. Throughout the growth cycle, strain 9117 showed less activity

than the original strain ATCC 29178 and the activity of the hemolysin corresponds to the growth of each individual strain.

The size of the factor responsible for hemolysis is known to be greater than 12000 MW due to the fact that the hemolysin of interest was unable to penetrate the 12000 to 14000 MWCO dialysis tubing used at the onset of the experiment. This also addresses the question as to whether the hemolysin of interest is associated with the *lct*O gene possessed by *S. iniae*. The size of the natural *lct*O gene is 1212 bp, with a resultant protein 403 amino acids long as described using NCBI GenBank (http://www.ncbi.nlm.nih.gov/) accession numbers 2239171 for the natural *lct*O gene, and CAA68903 for the protein sequence. The MW of this protein is 44119.29 as determined using the program EditSeq ™ Expert Sequence Analysis Software. These findings are in concordance with the fact that the lctO protein is also unable to pass through a 12000 MW cut-off dialysis bag due to the large size of this protein.

Effects of heat treatment on the hemolysin did not demonstrate any major effect on activity until a temperature of 60°C was reached, at this temperature the hemolysin exhibited a 25% reduction in activity. The results found here beginning at 60°C up to 95°C also demonstrate an irregular pattern of activity loss, which was not what was expected. This is most likely due to the deviation seen between the absorbance readings from the duplicate samples used in this experiment. When samples were boiled a 41% loss of activity was observed, indicating that the heat was causing denaturization, possibly through the breakage of hydrogen bonds or other noncovalent bonds (35).

The hemolysin demonstrated reduced activity when incubated with the sterol cholesterol, which suggested that the hemolysin could be associated with the thiol-

activated toxins. Thiol-activated toxins are antigenically related hemolysins that have only been found in four genera of bacteria (*Clostridium, Listeria, Bacillus,* and *Streptococcus*) (41). These toxins all exhibit reversible loss of activity when exposed to oxygen and are irreversibly inhibited by small concentrations of cholesterol (10,41). However, due to the fact that this hemolysin does not exhibit loss of activity under aerobic conditions, this provided evidence that this hemolysin is not related to the thiol-activated toxins.

Hemolytic activity was also partially inhibited by proteinase K, suggesting that the hemolysin may be a protein or has an essential protein component required for activity. Proteinase K has a broad specificity of target sites, but it preferentially cleaves at sites which possess aromatic and large hydrophobic amino acids (46). In this particular study, it is possible that the loss of activity observed could be due to the heat exposure the sample was subject to in order to inactivate the proteinase K enzyme during the course of the experiment. The hemolysin also demonstrated partial loss of activity when incubated with the enzyme lipase. This enzyme (when found in the human pancreas) targets triacylglycerols and hydrolyses them, thereby playing a very important role in fat absorption (53). The fact that the factor of interest exhibited loss of activity when incubated with this enzyme can be explained by the fact that in a previous protease assay, this particular bottle of lipase had demonstrated proteolytic activity (29).

The activity of the hemolysin was influenced by the presence of trypsin. The fact that incubation with trypsin resulted in a higher titre than the untreated material suggests that the trypsin had hemolytic activity on it's own. However, during the course of this study activity levels were not assessed on trypsin alone. Trypsin is a serine protease

enzyme, the function of which is to cleave sites just past the carbonyl groups of lysine or arginine residues and results in the degradation of the protein into peptide fragments (9). Characterization using trypsin helps to determine whether carbonyl groups are present within the protein (54). The presence of either proline or acidic amino acids at the usual cleavage site can make the peptide bonds resistant to cleavage by this enzyme (46). Also noted during this experiment was the fact that the effect of increased temperatures on the sample results in a decrease in activity. This suggests that the influence on hemolytic activity demonstrated by trypsin could be even higher than what was found in this experiment because the increase in temperature should have decreased the activity of the hemolysin.

Non-denaturing PAGE analysis along with the gel overlay hemolysin detection was conducted in order to separate the various proteins present in the culture supernatant, and in an attempt to assess whether one of these bands corresponded with a hemolytic band produced by an analogous gel overlaid onto a blood agar plate. Results from this assay indicated a zone of hemolysis on the blood agar plate. However, it was noted that the sample buffer used to load the sample on the gel demonstrated an identical zone of hemolysis. No other zone of hemolysis was evident, suggesting what was observed was non-specific. Further analysis with more concentrated samples and different buffer systems would be required for detection of this *S. iniae* hemolytic activity.

CONCLUSION

In conclusion, a technique for introducing exogenous DNA into *S. iniae* was described. This involved determination of the best type of broth to grow the organism in, the best time at which to transform, and the best type of electrotransformation wash solution to use. However, the transformation efficiency obtained during this experiment was very low. The low transformation efficiency seen in this study is not surprising. Electrotransformation is still a relatively new concept in molecular biology and involves a number of different parameters to obtain the optimal conditions. These parameters are not only based on the conditions determined, but on the type of organism to be transformed as well. Gram-positive organisms are known to be more resistant to physical stresses due to the thick outer wall they possess (48). This resistance can also be applied to electrotransformation, which makes gram-positive organisms more difficult to transform than gram-negative organisms.

The conditions determined here play a very important role by providing a starting point toward development of a higher efficiency transformation procedure in the future. Moreover, from the results obtained it could not be conclusively ascertained whether the *lct*O gene is associated with hemolysis.

Preliminary characterization of the hemolysin showed that the hemolysin expressed by *S. iniae* (ATCC 29178) is found at the beginning of the exponential phase of growth and is expressed much earlier and at higher levels than another strain of *S. iniae* (9117). Further characterization revealed partial loss of activity after treatment with proteinase K and cholesterol, and an increased activity level was found after treatment

with trypsin. It is unclear at this time however, if trypsin had any hemolytic activity on its own. The factor of interest also appears to have a molecular weight greater than 12000 daltons. During the course of this study, the hemolysin produced by *S. iniae* proved to be somewhat difficult to interpret. It is not known at this time whether this hemolysin is a single entity or if it is a group of molecules, which would possibly explain the difficulty of gathering data from some of the experiments performed. Characterization studies of the hemolysin must be considered as preliminary data at this time. Information on electrotransformation can be utilized as a starting point to improve transformation efficiency in this organism. Both aspects can be used to help further characterize and study the role of the hemolysin in the virulence of *S. iniae*.

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