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Streptococcus iniae Capsule Impairs Phagocytic Clearance and Contributes to Virulence in Fish▽

Jeffrey B. Locke,[1,2]† Kelly M. Colvin,[4]† Anup K. Datta,[3] Silpa K. Patel,[1] Nandita N. Naidu,[3] Melody N. Neely,[5] Victor Nizet,[1,2,3] and John T. Buchanan[1,4]*

Department of Pediatrics, Division of Pharmacology and Drug Discovery,[1] Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography,[2] and Glycotechnology Core Resource, Glycobiology Research and Training Center,[3] University of California, San Diego, La Jolla, California 92093; Kent SeaTech Corporation, San Diego, California 92121[4]; and Department of Immunology and Microbiology, Wayne State School of Medicine, Detroit, Michigan 48201[5]

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Surface capsular polysaccharides play a critical role in protecting several pathogenic microbes against innate host defenses during infection. Little is known about virulence mechanisms of the fish pathogen *Streptococcus iniae*, though indirect evidence suggests that capsule could represent an important factor. The putative *S. iniae* capsule operon contains a homologue of the *cpsD* gene, which is required for capsule polymerization and export in group B *Streptococcus* and *Streptococcus pneumoniae*. To elucidate the role of capsule in the *S. iniae* infectious process, we deleted *cpsD* from the genomes of two virulent *S. iniae* strains by allelic exchange mutagenesis to generate the isogenic capsule-deficient Δ*cpsD* strains. Compared to wild-type *S. iniae*, the Δ*cpsD* mutants had a predicted reduction in buoyancy and cell surface negative charge. Transmission electron microscopy confirmed a decrease in the abundance of extracellular capsular polysaccharide. Gas-liquid chromatography–mass spectrometry analysis of the *S. iniae* extracellular polysaccharides showed the presence of L-fucose, D-mannose, D-galactose, D-glucose, D-glucuronic acid, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine, and all except mannose were reduced in concentration in the isogenic mutant. The Δ*cpsD* mutants were highly attenuated in vivo in a hybrid striped bass infection challenge despite being more adherent and invasive to fish epithelial cells and more resistant to cationic antimicrobial peptides than wild-type *S. iniae*. Increased susceptibility of the *S. iniae* Δ*cpsD* mutants to phagocytic killing in whole fish blood and by a fish macrophage cell line confirmed the role of capsule in virulence and highlighted its antiphagocytic function. In summary, we report a genetically defined study on the role of capsule in *S. iniae* virulence and provide preliminary analysis of *S. iniae* capsular polysaccharide sugar components.

Streptococcus iniae was first isolated from an Amazon River dolphin (*Inia geoffrensis*) in the 1970s (38). Though *S. iniae* infections in humans can occur in the form of cellulitis resulting from a fish handling injury (52), this bacterium is primarily problematic as an aquatic pathogen. Over 30 freshwater and saltwater fish species have demonstrated susceptibility to the disease, including such economically important species as tilapia (39), yellowtail (26), trout (16), and hybrid striped bass (HSB) (17). Common clinical symptoms of *S. iniae* infection in fish include loss of orientation, lethargy, ulcers, exophthalmia, and erratic swimming (6). Mortality resulting from *S. iniae* infection is often attributed to meningoencephalitis and is responsible for aquaculture losses measured in the hundreds of millions of dollars annually. *S. iniae* can also cause significant disease outbreaks in wild fish populations (53). The virulence mechanisms of *S. iniae* are largely unknown.

Our preliminary screening of an *S. iniae* transposon mutant library in HSB indicated that genes involved in capsule synthesis may be associated with virulence. Among other genes, we found that the disruption of the phosphoglucomutase gene resulted in a putative alteration of cell wall architecture, capsule expression, and virulence attenuation associated with increased susceptibility to antimicrobial peptides (AMPs) compared to wild-type (WT) *S. iniae* in an HSB infection challenge (8). A similar *S. iniae* transposon library screen in zebrafish revealed that mutations leading to decreased buoyancy (reflective of potential defects in capsule synthesis) represented a significant proportion of the attenuated mutants (31).

The molecular basis for capsule synthesis has been studied in several streptococcal species (29). For *S. iniae*, electron micrographs show the presence of an extracellular capsule (3, 8), and a putative capsule operon sequence has been identified (GenBank accession no. AY904444). Multiple streptococcal capsule operons contain a conserved group of genes (*cpsA* to *-E*) that are collectively responsible for capsule chain length determination and export (11, 21, 32). The *cpsD* gene, encoding an autophosphorylating protein tyrosine kinase, has been identified as required for capsule synthesis in *Streptococcus pneumoniae* (4, 33). In group B *Streptococcus* (GBS) (*S. agalactiae*), allelic replacement of *cpsD* resulted in a 91% reduction in capsular polysaccharide (11). Blast analysis (1) of the predicted amino acids of the *S. iniae* CpsD homologue (GenBank accession no. AAY17296) showed that it has 63% identity and 82% similarity to GBS CpsD. To gain a better understanding of the potential role of capsule in *S. iniae* infection, we deleted the *S.*

* Corresponding author. Mailing address: University of California, San Diego, School of Medicine, 9500 Gilman Drive, Mail Code 0687, La Jolla, CA 92093. Phone: (858) 534-9760. Fax: (858) 534-5611. E-mail: jtbuchan@ucsd.edu.

† These authors contributed equally to the research presented in this publication.

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iniae cpsD homologue and compared the resulting isogenic mutant to the WT parent strain by using biochemical techniques and a series of in vitro and in vivo models of disease pathogenesis.

MATERIALS AND METHODS

Bacterial strains, culture, transformation, and DNA techniques. WT *S. iniae* strain K288 was isolated from the brain of a diseased HSB at the Kent SeaTech aquaculture facility in Mecca, CA (8). *S. iniae* strain 94-426 was originally isolated from a diseased tilapia. All *S. iniae* strains were grown at 30°C (unless otherwise stated) in Todd-Hewitt broth (THB) (Hardy Diagnostics) or Todd-Hewitt agar (THA). Enumeration of CFU for in vitro assays and in vivo infections was performed by plating dilutions on THA. Beta-hemolytic activity of *S. iniae* was assessed on sheep blood agar plates (tryptic soy agar with 5% sheep red blood cells added). In all assays, overnight cultures of *S. iniae* were diluted 1:10 in fresh THB and grown to mid-log phase (optical density at 600 nm of 0.40). *S. iniae* strains were rendered electrocompetent for transformation through growth in THB containing 0.6% glycine according to procedures described for GBS (18); transformants were propagated at 30°C in THB with 0.25 M sucrose. Antibiotic selection was achieved with chloramphenicol (Cm) at 4 μg/ml, erythromycin (Erm) at 5 μg/ml, or spectinomycin at 100 μg/ml. *Escherichia coli* used in cloning was grown at 37°C (unless otherwise stated), with shaking, under aerobic conditions in Luria-Bertani broth (Hardy Diagnostics) or statically on Luria agar. When necessary, *E. coli* was grown in antibiotics, i.e., ampicillin at 100 μg/ml, spectinomycin at 100 μg/ml, Erm at 500 μg/ml, or Cm at 20 μg/ml. Mach1 chemically competent *E. coli* (Invitrogen) and MC1061 electrocompetent *E. coli* used in transformations were recovered through growth at 30°C in SOC medium (Invitrogen). A PureLink Quick plasmid miniprep kit (Invitrogen) was used to isolate plasmids propagated in *E. coli*. *S. iniae* genomic DNA was isolated using the UltraClean DNA isolation kit (MoBio).

Cell lines and culture conditions. The adherent carp monocytic/macrophage cell (CLC) line (European Collection of Cell Cultures 95070628) and the WBE27 white bass embryonic epithelial cell line (ATCC CRL-2773) (48) were grown at 28°C with 5% CO_2. Cells were passaged fewer than 10 times and maintained in 125-ml tissue culture flasks in Dulbecco modified Eagle medium (DMEM)(Gibco) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco).

Statistical analyses. Data analyses were performed using the statistical tools included with GraphPad Prism (GraphPad Software, Inc.). In vitro assay data were analyzed using unpaired two-tailed *t* tests. Fish infection survival data were analyzed using a log rank test. A P value of <0.05 was considered statistically significant. In vitro assays were repeated three times, in quadruplicate, and the data presented (means ± standard errors of the means [SEM]) are from a single representative assay.

Allelic exchange mutagenesis. Allelic exchange mutagenesis of *S. iniae* strains K288 and 94-426 was carried out as previously described (7), with the only significant modification being the use of the Gateway cloning system (Invitrogen). For PCR, all primers were designed based on the *cpsD* gene region of the *S. iniae* capsule operon deposited under GenBank sequence accession number AY904444. PCR was used to amplify ~400 bp of *S. iniae* chromosomal DNA fragments directly upstream and downstream of *cpsD*, with primers adjacent to *cpsD* constructed to possess 25-bp 5′ extensions corresponding to the 5′ and 3′ ends of the chloramphenicol acetyltransferase (*cat*) gene from pACYC (34), respectively. The upstream and downstream PCR products were then combined with a 660-bp amplicon of the complete *cat* gene by using fusion PCR (51). The resultant PCR amplicon containing an in-frame substitution of *cpsD* with *cat* was subcloned into the Gateway entry vector pCR 8/GW/TOPO and transformed into chemically competent Mach1 *E. coli* (Invitrogen). Plasmid DNA was extracted, and a Gateway LR recombination reaction was performed to transfer the fusion PCR amplicon into the corresponding Gateway entry site of a temperature-sensitive knockout vector, pKODestErm (created for Gateway cloning from pHY304 (9), to generate the knockout plasmid pKOcpsD. Following propagation in MC1061 *E. coli*, the pKOcpsD construct was introduced into WT *S. iniae* by electroporation. Transformants were identified at 30°C by Erm selection and shifted to the nonpermissive temperature for plasmid replication (37°C). Differential antibiotic selection (Cm^r and Erm^s) was used to identify candidate allelic exchange mutants. Targeted in-frame replacement of *cpsD* was confirmed unambiguously by PCRs documenting the desired insertion of *cat* and absence of *cpsD* sequence in chromosomal DNA isolated from both of the final Δ *cpsD* mutants and by phenotype, with the observation of rapid sinking in liquid culture.

Transmission electron microscopy. Capsular polysaccharide of mid-log-phase WT K288 and K288 Δ*cpsD* was visualized via transmission electron microscopy using a lysine acetate fixation protocol as previously described (23). The only notable deviation in this protocol was the use of an overnight room temperature incubation in the second fixation step. Samples were embedded in LR White (Fluka), sectioned, and counterstained with uranyl acetate. Grids were viewed and photographed using a JEOL 1200EX II transmission electron microscope (JEOL, Peabody, MA) at a magnification of ×15,500 and an acceleration voltage of 80 kV.

Cytochrome *c* assay. Anionic cell surface charge was measured through a cytochrome *c* binding assay as previously described (8). An overnight culture of each *S. iniae* strain was diluted 1:10 and grown to mid-log phase. Five milliliters of the bacteria was pelleted at 13,000 × *g* for 5 min and resuspended in 1 ml of MOPS (morpholinepropanesulfonic acid) (pH 7.0). The bacteria were pelleted and then resuspended in 450 μl of MOPS and 50 μl of 10-mg/ml cytochrome *c* (Sigma). The solution was vortexed and incubated at room temperature for 15 min. The bacteria were pelleted, and 200 μl of the supernatant was added to a flat-bottom 96-well plate. The amount of unbound cytochrome *c* was determined by absorbance of the supernatant at 530 nm.

Growth rate analysis and hemolytic activity. Mid-log-phase cultures of WT *S. iniae* and the Δ*cpsD* mutant were diluted 1:10 in a 96-well plate. Growth was monitored via optical density readings at 600 nm, in quadruplicate, every 30 min for 8 h. Hemolytic activity against sheep red blood cells was measured as described previously (19).

Invasion and adherence assays. Invasion and adherence assays were performed in collagenized 96-well tissue culture plates (Costar). White bass epithelial cells (WBE27) were seeded at a density of 1×10^5 cells per well and allowed to grow overnight. The medium was replaced with 100 μl DMEM containing 2% FBS. Bacteria from a mid-log-phase culture were diluted in DMEM with 2% heat-inactivated FBS, and 100 μl was added to achieve a multiplicity of infection (MOI) of 10 (bacteria to cells). Following centrifugation at 350 × *g* for 5 min, the plate was incubated for 1 h at 28°C with 5% CO_2. The cells were washed three times with DMEM and incubated in fresh DMEM with 20 μg/ml penicillin (Invitrogen) and 200 μg/ml of gentamicin (Invitrogen) for 2 h to kill extracellular bacteria. Cells were then washed three times with phosphate-buffered saline (PBS) and lysed by trituration in 100 μl of 0.01% Triton X-100 (Sigma). Surviving intracellular bacteria were quantified by plating serial dilutions of lysed cell supernatant on THA. Adherence assays were carried out in a similar manner except that no antibiotics were used and the bacteria were incubated with the cells for 30 min and washed five times with PBS to remove nonadherent bacteria prior to enumeration of CFU.

Capsular polysaccharide isolation and purification. Capsular polysaccharide was extracted from 2 liters of 94-426 *S. iniae* culture by using methods described for other encapsulated species (20). Briefly, overnight cultures were treated with a final concentration of 1% Cetavlon, a polycationic detergent that precipitates polyanionic polysaccharides. The precipitate was collected by centrifugation and resuspended in water, and $CaCl_2$ added to a final concentration of 1 mM to separate polysaccharide from detergent. Nucleic acids were precipitated from solution by adding 25% (vol/vol) ethanol, followed by centrifugation. Capsule in the supernatant was subsequently precipitated by ethanol at a final concentration of 80% (vol/vol). Contaminating protein, traces of Cetavlon, and other low-molecular-mass contaminants were removed with proteinase K digestion and extensive dialysis against a buffer composed of 10% ethanol, 50 mM NaCl, and 5 mM Tris. Capsule was further purified with a Sephacryl 200 gel filtration column using 50 mM ammonium formate elutions. Column fractions were tested for neutral sugar estimation by phenol sulfuric acid assay (14). Void-volume fractions were pooled and concentrated by speed vacuuming and analyzed by deoxycholate-polyacrylamide gel electrophoresis (42) and Alcian blue staining (42).

Glycosyl composition analysis. The glycosyl composition of capsular polysaccharide was determined by the preparation and analysis of trimethylsilyl methylglycosides (40). Briefly, samples were methanolyzed with 1 M methanolic HCl at 80°C for 18 h, followed by re-N-acetylation of methylglycosides by use of pyridine-acetic anhydride in the presence of methanol at 100°C for 1 h. The free hydroxyl groups of re-N-acetylated methylglycosides were trimethylsilylated using Tri-Sil reagent (Pierce) at 80°C for 20 min. The volatile trimethylsilyl methylglycosides were then analyzed by combined gas-liquid chromatography–mass spectrometry (GLC-MS) using a DB-1 capillary column (J&W Scientific) (30 m by 0.25 mm), and detection was done with a mass selective detector (Hewlett-Packard HP 5890 series II GC interfaced to a 5971A mass selective detector).

In vivo fish challenges. Groups of 20 (~40-g) HSB (*Morone chrysops* × *Morone saxatilis*) were used for in vivo infection studies. Fish were maintained at 25°C in ~75-liter flowthrough tanks. An overnight culture of each *S. iniae* strain

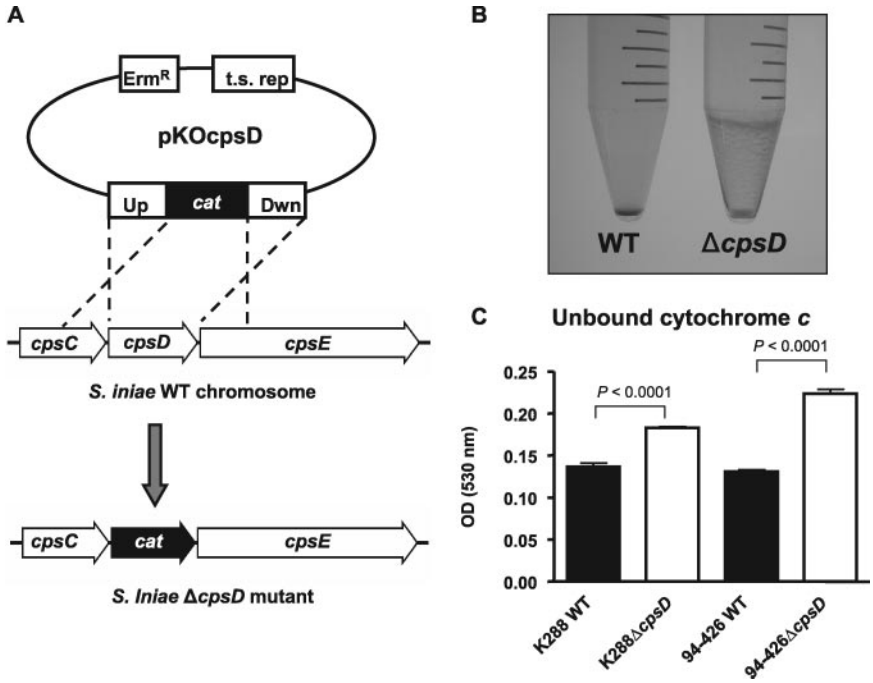

FIG. 1. Allelic exchange mutagenesis of *S. iniae cpsD* results in a capsule-deficient phenotype. (A) A knockout plasmid, pKOcpsD, was created, containing erythromycin resistance (Erm^R), a temperature-sensitive origin of replication (t.s. rep), and a chloramphenicol resistance gene (*cat*) flanked by homologous regions of DNA upstream (Up) and downstream (Dwn) of *cpsD*. The knockout plasmid was used for precise in-frame replacement of the *S. iniae cpsD* gene with *cat*. (B and C) Allelic replacement of *cpsD* resulted in reduced capsule production as seen by reduced buoyancy in liquid culture (B) and a reduction in negative cell surface charge measured indirectly through amount of positively charged cytochrome *c* remaining unbound to the bacteria (mean ± SEM) (C). OD, optical density.

was diluted 1:10 and grown to mid-log phase. The bacteria were pelleted, resuspended in PBS, and diluted appropriately to deliver the desired dose in a 100-μl intraperitoneal (i.p.) injection. Survival was monitored for 7 days. Fish challenges were carried out in an ALAAC-certified facility following IACUC-approved protocols.

Survival in whole blood. Blood was extracted via syringe from the caudal vein of three HSB and collected in a heparinized tube. Three hundred microliters of each blood sample was immediately added to two 2-ml siliconized microcentrifuge tubes with approximately 300 CFU of mid-log-phase bacteria. The tubes were incubated with shaking at 30°C for 1 h. Two 100-μl aliquots from each blood sample were spread onto THA to enumerate surviving bacteria. Survival was calculated as a percentage of remaining bacteria relative to the starting inoculum.

Total cell killing and intracellular survival. Total phagocytic survival assays were carried similarly to invasion and adherence assays. Bacteria were incubated with CLCs at an MOI of 0.1. Cells were lysed and plated as described above for invasion and adherence assays. Survival is expressed as CFU per ml of lysed cell supernatant at each time point. Intracellular growth assays were carried out in a manner similar to that for entry assays. Bacteria were incubated with the CLCs at 28°C at an MOI of 10. After 1 h, the medium was replaced with fresh DMEM with 20 μg/ml penicillin (Invitrogen) and 200 μg/ml of gentamicin (Invitrogen) to kill extracellular bacteria. After 4 h in antibiotics, the medium was replaced with fresh DMEM containing 2% FBS. The cells were lysed and plated to determine surviving CFU as described above. Survival is expressed as CFU per ml of lysed cell supernatant at each time point. For a visual comparison of phagocytosis in CLCs, bacteria were labeled by being grown to an optical density at 600 nm of 0.40 in THB plus 50 μg/ml fluorescein isothiocyanate (FITC) (Molecular Probes). Bacteria were washed twice in PBS and added at an MOI of 10 to CLC monolayers as described above. After incubation with antibiotics as described above to kill extracellular bacteria, monolayers were washed two times with PBS, and SYTOX Orange (Molecular Probes) was added to a final concentration of 0.5 μM to each well. Bacteria were visualized with a Zeiss Axiovert 100 inverted microscope with appropriate fluorescent filters. FITC-labeled intracellular bacteria appeared green, and remaining extracellular bacteria killed by antibiotics were labeled with SYTOX Orange and appeared red. Images were captured with a charge-coupled device camera using the Axiovision software package (Zeiss).

Resistance to AMPs. Mid-log-phase cultures of *S. iniae* were diluted in fresh THB to ~3×10^4 CFU/ml, and 180 μl of this bacterial suspension was added to wells of a 96-well plate. Dilutions of the antimicrobial peptides moronecidin (28) (1.5 μM final concentration in the well) and polymyxin B (Sigma) (60 μM final concentration in the well) were prepared in distilled water and added to wells in 20-μl volumes; distilled water alone was used as a control. To measure antimicrobial killing kinetics, 20-μl aliquots from each well were serially diluted in PBS and plated at specified time points after addition of the antimicrobial peptide for CFU determination. Each treatment was performed in four replicate wells. Kinetic killing data were calculated for each time point by dividing the treatment group CFU by the control CFU.

Oxidant susceptibility assay. Bacterial strains were grown to mid-log phase and diluted 1:10 in PBS, and 100 μl was added to a 96-well plate, resulting in ~3×10^6 CFU/well. Hydrogen peroxide (H_2O_2) (Fisher Scientific) was added to a 0.035% final concentration. Bacteria were incubated at 30°C, and the reaction was quenched at time end points by adding 1,000 U of catalase (Sigma). Dilutions were plated on THA to determine the number of surviving CFU.

RESULTS

Mutagenesis of *S. iniae cpsD* reduces surface capsular polysaccharide. Precise, in-frame allelic replacement of the *cpsD* gene was achieved in *S. iniae* strains K288 and 94-426 to create K288 Δ*cpsD* and 94-426 Δ*cpsD* (Fig. 1A). Each *S. iniae* Δ*cpsD* allelic replacement mutant exhibited reduced buoyancy in liquid culture (Fig. 1B). Loss of capsule was corroborated by loss of anionic charge on the surface of Δ*cpsD* mutants, as determined by decreased cytochrome *c* binding (36) (Fig. 1C). As reported for capsule mutants of other streptococci (15, 31) and also observed in our observations of increased chain length in GBS strain COH1 Δ*cpsE* isogenic capsule mutants (44) (data not shown), the *S. iniae* Δ*cpsD* mutants formed chains of

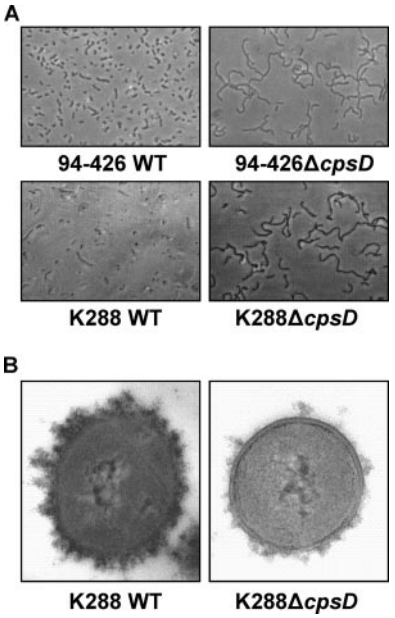

FIG. 2. Deletion of *cpsD* increases coccus chain length and reduces extracellular capsular polysaccharide. (A) Bright-field microscopy (magnification, ×400) reveals increased coccus chain length of the Δ*cpsD* mutants compared to WT *S. iniae*. (B) Transmission electron microscopy (magnification, ×15,500) shows a decrease in capsule in the K288 Δ*cpsD* mutant compared to WT K288.

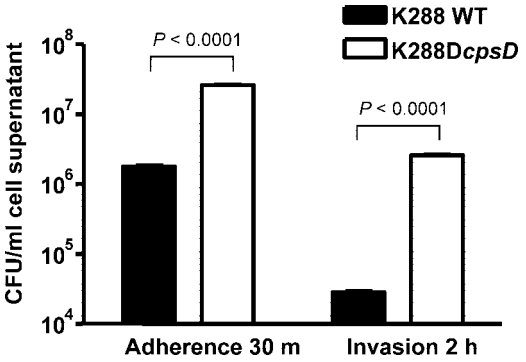

FIG. 3. Reduction in capsule increases adherent and invasive properties of *S. iniae*. *S. iniae* strain K288 was less adherent and invasive than capsule-deficient strain K288 Δ*cpsD* after incubation with WBE27 white bass epithelial cells. Adherent bacteria were enumerated after 30 min, and invasive intracellular bacteria were enumerated at 2 h (mean ± SEM). Similar results were observed for WT strain 94-426 and it Δ*cpsD* mutant.

greater length than the WT parent strains (Fig. 2A). Compared to the wild type, a clear reduction in surface capsular polysaccharide in the K288 Δ*cpsD* mutant was visualized through transmission electron microscopy (Fig. 2B). The Δ*cpsD* mutants had identical growth rates and similar hemolytic activity to the WT *S. iniae* strains (data not shown). It should be noted that complementation of the K288 Δ*cpsD* mutant was attempted by cloning the *S. iniae cpsD* gene into the multiple cloning sites of the constitutive high-expression plasmid pDCerm (25) and the tetracycline-inducible expression plasmid pLR16T (41). Expression of *cpsD* in pDCerm did not restore a wild-type phenotype; however, complementation with pLR16T (over a tight range of tetracycline levels) resulted in partial restoration of WT liquid culture buoyancy and coccus chain length phenotypes to the K288 Δ*cpsD* mutant (data not shown).

***S. iniae* capsule mutants show increased epithelial cell adherence and invasion.** A frequent observation in capsule-deficient streptococci is an enhancement of cellular adherence and invasion compared to those of WT strains (23, 35). Consistent with this pattern, the Δ*cpsD* mutants displayed a ~10-fold increase in adherence and a ~100-fold increase in intracellular invasion of cultured white bass epithelial cells compared to the parent strains ($P < 0.0001$) (Fig. 3).

***S. iniae* capsular sugars are reduced in the Δ*cpsD* mutant.** GLC-MS composition analysis of a capsular monosaccharide preparation revealed that the capsule of WT *S. iniae* strain 94-426 potentially contains L-fucose, D-mannose, D-galactose, D-glucose, D-glucuronic acid, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine (Table 1). It is possible that some of these sugars exist in noncapsular polysaccharides. We found a significant reduction in putative capsular monosaccharides, with the exception of D-mannose, in the capsule-deficient Δ*cpsD* *S. iniae* isogenic mutant (Fig. 4). Given its relative abundance in both WT and Δ*cpsD* *S. iniae*, it is possible that D-mannose exists as a noncapsular, cell surface polysaccharide component.

Reduction of capsule attenuates *S. iniae* infection in hybrid striped bass. The effect of the Δ*cpsD* mutation on *S. iniae* virulence was assessed through an i.p. infection challenge in HSB (Fig. 5A and B). Both WT *S. iniae* strains resulted in 100% HSB mortality within 1 week at an inoculum of 3×10^6 CFU. In contrast, injections of up to 100-fold-greater inocula of the respective Δ*cpsD* mutants caused no mortality or visual signs of infection in the fish.

***S. iniae* capsule promotes resistance to whole-blood and macrophage killing.** To elucidate potential mechanisms for the observed in vivo attenuation of the capsule mutants, *S. iniae* survival in fresh whole HSB blood was measured. Both of the *S. iniae* Δ*cpsD* mutants were significantly more susceptible to blood killing than the WT strains (Fig. 6A), indicating increased clearance by innate immune defenses. To further assess the role of the capsule in promoting *S. iniae* survival, WT *S. iniae* and the isogenic Δ*cpsD* mutant strains were incubated with a cultured fish macrophage cell line. The capsule-deficient mutant was over 20-fold more susceptible to killing by the macrophages in the in vitro assay ($P < 0.0001$) (Fig. 6B).

TABLE 1. Monosaccharide components of *S. iniae* strain 94-426 extracellular polysaccharide

Monosaccharide	mol % in:	
	WT	Δ*cpsD* mutant
L-Fucopyranose	7.90	2.20
D-Mannopyranose	20.26	67.50
D-Galactopyranose	24.70	10.12
D-Glucopyranose	21.60	9.12
D-Glucuronic acid	10.40	4.30
N-Acetyl-D-galactosamine	7.30	3.50
N-Acetyl-D-glucosamine	7.40	2.0

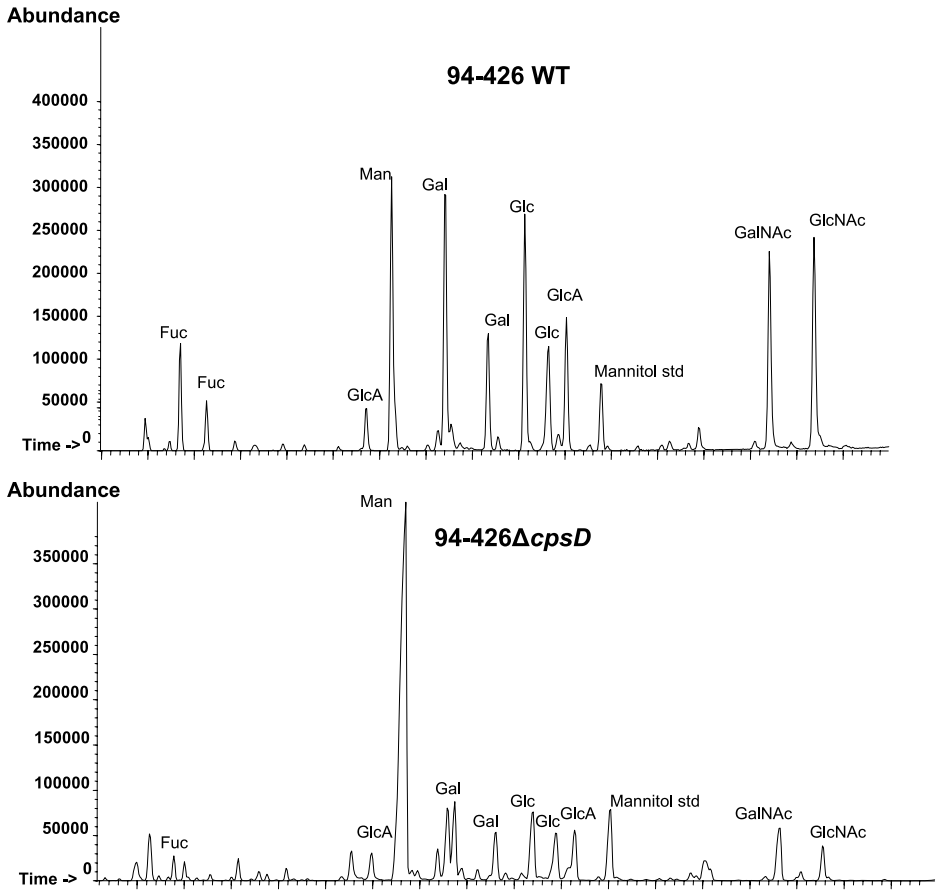

FIG. 4. GLC-MS analysis of *S. iniae* extracellular polysaccharides shows a reduction in 94-426 Δ*cpsD* monosaccharides compared to wild type. GLC-MS elution spectra after capsular preparation from on overnight culture of *S. iniae* are shown. The spectra define the potential capsular monosaccharides of *S. iniae* and indicate a significantly decreased abundance of capsular monosaccharides in the 94-426 Δ*cpsD* capsule-deficient mutant compared to the wild type. Sugar abbreviations: Fuc, L-fucose; Man, D-mannose; Gal, D-galactose; Glc, D-glucose; GlcA, D-glucuronic acid; GalNAc, *N*-acetyl-D-galactosamine; and GlcNAc, *N*-acetyl-D-glucosamine.

An *S. iniae* capsule mutant is less susceptible to cationic AMPs. One evolutionarily conserved mechanism for innate immune defense against bacterial infection is the production of cationic AMPs by phagocytes and other host cell types. We compared the susceptibilities of both WT *S. iniae* strains and the Δ*cpsD* isogenic mutants to the HSB AMP moronecidin and found the mutant strain to exhibit significantly ($P < 0.0001$) delayed killing kinetics (i.e., increased resistance) (Fig. 7A). Similar differences ($P < 0.001$) were observed in parallel assays performed with the bacterially derived cationic AMP polymyxin B (Fig. 7B). These studies indicate that the susceptibility to whole-blood and macrophage killing of the capsule-deficient strains does not derive from enhanced sensitivity to AMPs.

Loss of *S. iniae* capsule expression does not affect hydrogen peroxide sensitivity. An additional mechanism for phagocyte control of bacterial pathogens is reactive oxygen species generated through the oxidative burst. We compared the sensitivities of WT *S. iniae* strains and the isogenic mutants to killing by hydrogen peroxide and observed no biologically significant differences (Fig. 7C), suggesting that avoidance of oxidant killing mechanisms does not explain the contribution of capsule to *S. iniae* survival in the whole-blood and macrophage killing assays.

Capsular polysaccharide expression by *S. iniae* impedes phagocytotic uptake. Further investigations were performed to determine the step at which *S. iniae* capsule expression interfered with phagocyte killing, using a cultured fish macrophage cell line. The macrophages bound (Fig. 8A) and internalized (Fig. 8B) the capsule-deficient mutants much more efficiently than WT *S. iniae*. Upon phagocytosis by the macrophages, both WT and Δ*cpsD* mutant strains were rapidly and effectively killed intracellularly (Fig. 8C). Thus, the contribution of the *S. iniae* capsule to resisting phagocytic clearance was through impeding phagocytosis, not enhancing intracellular survival.

DISCUSSION

Capsule is an important extracellular feature of many bacterial species, with functions including protection against desiccation, adherence to host tissues, and resistance to both innate and adaptive host defenses (43). The extracellular polysaccharide capsules of several pathogenic streptococci have been established as virulence factors (29), acting through mechanisms including molecular mimicry (12), resistance to complement-mediated killing (13, 30), antigenic variation (5, 10), and impairment of phagocytosis (2, 47, 49). Here we used

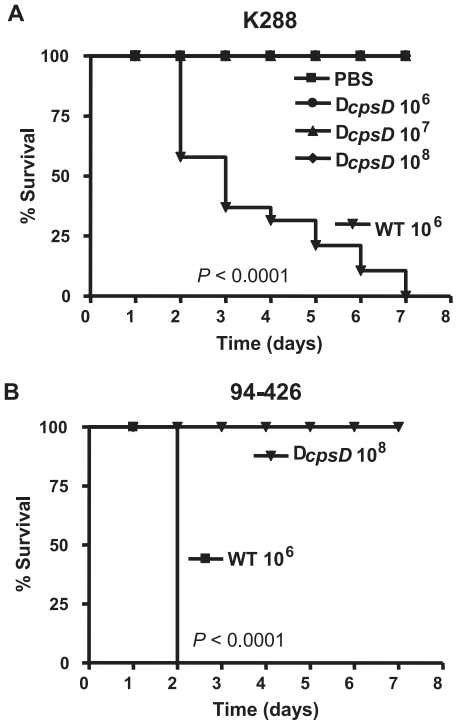

FIG. 5. Capsule contributes to *S. iniae* virulence in vivo as indicated in Kaplan-Meier survival plots showing attenuation of the Δ*cpsD* mutants. (A) Hybrid striped bass (groups of 20) were injected intraperitoneally with 3×10^6 CFU of WT K288; with 3×10^6, 10^7, or 10^8 CFU of the K288 Δ*cpsD* mutant; or with PBS. (B) Hybrid striped bass (groups of 20) were injected intraperitoneally with 3×10^6 CFU of WT 94-426 or with 3×10^6 CFU of the 94-426 Δ*cpsD* mutant. Mortality (100%) was observed only in the WT-injected fish for each strain.

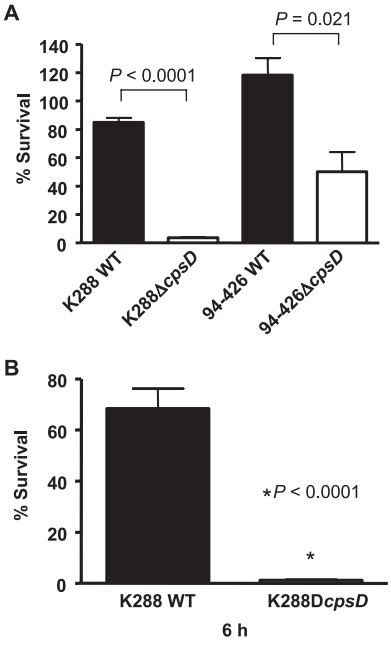

FIG. 6. *S. iniae* capsule decreases susceptibility to killing by whole blood and macrophages. (A) Survival of capsule-deficient Δ*cpsD* mutants is significantly decreased compared to that of wild-type *S. iniae* following 1 h of incubation in fresh whole fish blood. (B) K288 Δ*cpsD* is more sensitive than wild-type *S. iniae* to total phagocytic killing after a 6-h incubation with CLCs. Data are presented as mean ± SEM.

allelic replacement mutagenesis to provide evidence of a gene (*cpsD*) required for *S. iniae* capsule synthesis and a genetically defined study of the virulence role of capsule in this important leading aquaculture pathogen.

Allelic exchange mutagenesis of *cpsD* in two virulent *S. iniae* strains resulted in a capsule-deficient phenotype, with characteristics similar to those of capsule mutants of other streptococcal species, such as reduced negative cell surface charge, reduced buoyancy in liquid culture, and elongated coccus chain morphology. Transmission electron microscopy supported these observations and revealed a clear reduction in cell surface capsular polysaccharide in the Δ*cpsD* mutant compared to the wild type. Likewise, as reported for capsule-deficient GBS (24) and *Streptococcus pyogenes* (46), the *S. iniae* Δ*cpsD* mutants had increased adherence to and invasion of epithelial cells. We were unable to achieve full complementation to the wild-type phenotype of the Δ*cpsD* mutant by return of the wild-type gene on a constitutive expression plasmid. This may not be surprising, however. *cpsD* homologues in other streptococci have been shown to be essential for capsule polymerization and export to the cell surface but also have been shown to play a complex regulatory role involving protein autophosphorylation, with phosphorylation state regulating capsule production (4, 33). Complementation of this gene is likely to be difficult, and to date none of the studies addressing the function of *cpsD* in other streptococci through mutagenesis have reported successful complementation of this gene (see references 4, 11, and 33, among others). Nonetheless, the lack of clear complementation data restricts us from definitive conclusions regarding the role of *cpsD* in capsule production and leads us to hypothesize that either (i) toxicity to the bacterial cells may result from CpsD overexpression; (ii) the stoichiometry of CpsD interactions with other gene products involved in capsule biosynthesis is delicate and overexpression may decrease capsule production; or (iii) our chromosomal mutation, though by sequencing appearing to represent a precise allelic replacement from ATG start codon to stop codon, could have unanticipated polar effects elsewhere in the capsule operon, resulting in a capsule-deficient phenotype.

It is interesting to note that the capsule-deficient *S. iniae* mutants are significantly more resistant to AMPs. It is thought that charge plays a role in the binding affinity of cationic AMPs to the generally anionic bacterial surfaces. Through cytochrome *c* binding affinity assays we demonstrated that the Δ*cpsD* capsule-deficient mutants have reduced net negative surface charge compared to WT *S. iniae*, as expected with the loss of anionic capsular sugars from the cell surface. In *S. pyogenes* and *Staphylococcus aureus*, an increase in negative surface charge due to the loss of teichoic acid D-alanylation resulted in increased susceptibility to AMPs (27, 37). A similar charge-related mechanism may explain our results.

Considerable work has been done to characterize the monosaccharide sugar components of other pathogenic streptococci; however, the individual sugars and repeating multimer units of *S. iniae* capsular polysaccharides are unknown. We performed GLC-MS analysis of *S. iniae* extracellular polysaccharides and found a variety of neutral and charged sugars,

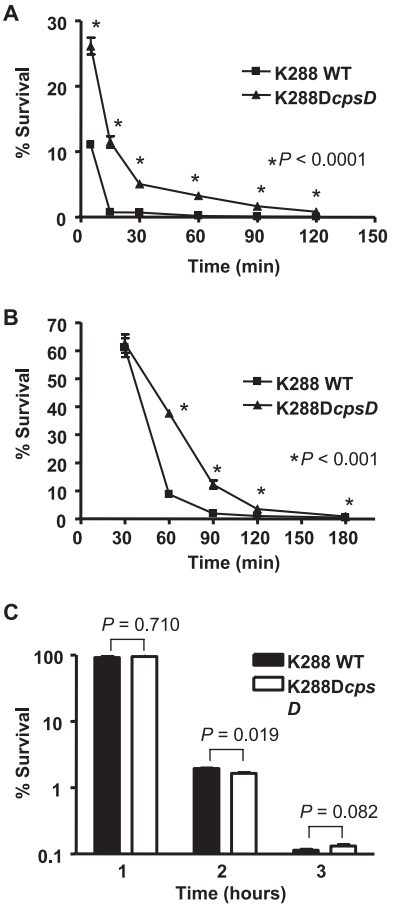

FIG. 7. Encapsulated *S. iniae* is more vulnerable to cationic antimicrobial peptides the Δ*cpsD* capsule mutants but equally susceptible to hydrogen peroxide killing. (A and B) Antimicrobial peptide kinetic killing profiles for 1.5 μM moronecidin (A) and 60 μM polymyxin B (B) indicate decreased sensitivity to AMPs for the K288 Δ*cpsD* mutant compared to wild-type K288 *S. iniae*. Similar results were observed for the 94-426 Δ*cpsD* mutant. (C) No biologically significant difference in survival of WT K288 and K288 Δ*cpsD* is observed following 1-, 2-, and 3-h incubations with hydrogen peroxide. Similar results were observed for the 94-426 Δ*cpsD* mutant. Data are presented as mean ± SEM.

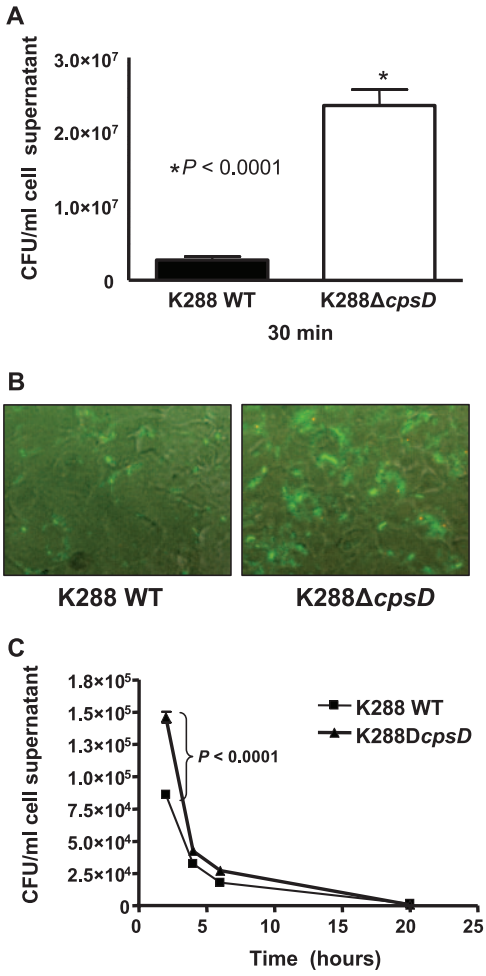

FIG. 8. Capsule hinders phagocytosis of *S. iniae* but does not confer intracellular protection against phagocytic killing. (A) The capsule-deficient K288 Δ*cpsD* mutant has increased binding affinity to the surface of CLC compared to WT K288 (mean ± SEM). (B) The K288 Δ*cpsD* mutant also is phagocytosed by CLCs more rapidly than WT K288. Fluorescence imaging (magnification, ×400) shows phagocytosed intracellular bacteria labeled with FITC (green), and adherent extracellular bacteria are labeled with SYTOX Orange (red). (C) Once phagocytosed, the K288 Δ*cpsD* mutant and WT K288 are both effectively killed over time as seen through enumeration of viable intracellular *S. iniae* in CLCs at 2, 4, 6, and 20 h postincubation (mean ± SEM).

indicating that *S. iniae* likely possesses a complex capsule structure. Based on the component sugars, capsules composed of hyaluronic acid, chondroitin, or heparin are possibilities, with the potential addition of neutral sugar side chains. Further analysis of the multimer subunits of *S. iniae* capsular polysaccharide will help to elucidate the role of *cpsD* in capsule synthesis. In GBS, CpsD functions in the later stages of capsular polysaccharide synthesis involving export of repeating units of sugars to the cell surface (11). In the *S. iniae* capsule-deficient mutants, we found intact capsular polysaccharide with all of the component sugars in roughly the same ratios; however, the amounts of these sugars were greatly reduced. In light of these data and the electron micrographs, we hypothesize that the *S. iniae* Δ *cpsD* mutants likely assemble the capsule polysaccharide repeating units but are deficient in their ability to express wild-type levels of capsular polysaccharides and export them to the cell surface.

The capsule-deficient Δ*cpsD* mutants of *S. iniae* proved to be highly attenuated in vivo, even when delivered at 100 times the 100% lethal dose for the WT. A reduction in virulence for capsule-deficient mutants has been demonstrated for several streptococcal species, including *S. suis* (49), GBS (45), and *S. pyogenes* (22, 46). In an effort to elucidate the mechanism by which capsule protects *S. iniae*, we noted that the capsule-deficient mutants were extremely sensitive to clearance in fresh HSB blood and in cell culture with fish macrophages. We documented that sensitivity to neither AMPs nor reactive oxygen species was increased in the capsule-deficient mutants. We did discover, however, a profound decrease in the ability of the capsule-deficient mutants to avoid binding and phagocytosis by fish macrophages. In *S. pneumoniae*, strains with increased negative surface charge due to the absence of choline-

binding proteins are significantly less adherent to human monocytes (50), suggesting that a major component of the increased binding affinity of capsule-deficient *S. iniae* to host cells may be related to loss of surface negative charge. Alternatively, another explanation for affinity towards host cells of unencapsulated streptococci could be the ability of capsule to mask surface-associated proteins or other factors that may play a role in host cell binding (24). Though *S. iniae* capsule potentially interferes with certain steps in the pathogenic process by decreasing attachment and invasion of epithelial cells, capsule simultaneously reduces the ability of host phagocytes to bind and phagocytose the bacterium. In our i.p. fish infection challenge, the latter phenomenon clearly plays the more critical role in determining the outcome of infection.

In summary, we present here proof that capsule is involved in *S. iniae* virulence. Through allelic replacement we have shown that *cpsD* is likely required for complete *S. iniae* capsule expression and that capsule plays a role in *S. iniae* virulence through its ability to lower the rate of phagocytosis by host immune cells. Our use of a natural host-pathogen infection challenge showed that the Δ*cpsD* mutants are over 100-fold attenuated compared to WT *S. iniae*, despite increased resistance to AMPs and increased adherence to and invasion of epithelial cells. Finally, we present preliminary data showing the individual monosaccharide components of *S. iniae* capsular polysaccharide. Having established capsule as a key *S. iniae* virulence determinant, further studies can explore the details of capsule synthesis and capsule regulation during various stages of the *S. iniae* infectious process.

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