Emergence of Novel *Streptococcus iniae* Exopolysaccharide-Producing Strains following Vaccination with Nonproducing Strains

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Received 15 April 2008/Accepted 10 September 2008

*Streptococcus iniae* is a major pathogen of fish, producing fatal disease among fish species living in very diverse environments. Recently, reoccurrences of disease outbreaks were recorded in rainbow trout (*Oncorhynchus mykiss*, Walbaum) farms where the entire fish population was routinely vaccinated. New strains are distinguished from previous strains by their ability to produce large amounts of extracellular polysaccharide that is released into the medium. Present findings indicate that the extracellular polysaccharide is a major antigenic factor, suggesting an evolutionary selection of strains capable of extracellular polysaccharide production.

*Streptococcus iniae* is an important fish pathogen associated with bacterial menigitis and sepsis of salmonids and other fish species both in the United States and in Israel (12). Accidental injuries of humans and subsequent contamination by strains associated with infected fish (but not environmental isolates) can also lead to human infections, mainly of the skin (15, 39).

Prior to 1998, the predominant *S. iniae* isolates were serotype I (arginine dehydroase [ADH]-positive, extracellular polysaccharide [EPS]-negative) strains. Routine vaccination against the type I strain (between 1995 and 1998) lead to the emergence of type II (ADH-negative, EPS-negative) strains that are capable of overcoming the fish immune response elicited by vaccination with *S. iniae* type I. Vaccine composition was therefore modified accordingly, now containing *S. iniae* KFP 173 type II (ADH-negative, EPS-negative). Due to legal constraints regarding autovaccines, *S. iniae* KFP 173 was subsequently replaced by another type II (ADH-negative, EPS-negative) strain, KFP 404. In 2005, reoccurrences of disease outbreaks were recorded in fish farms where the entire fish population was routinely vaccinated with the modified vaccine. Diseased fish showed major pathological changes in all internal organs, and pure colonies of beta-hemolytic *S. iniae* colonies (41), phenotypically undistinguishable from the previously described type II (ADH-negative) strains (obtained from the viscera of the diseased fish), were isolated on blood agar plates. Batch culture fermentation of vaccine escape isolates revealed that, contrary to previously described strains, new strains gave rise to a viscous culture, resembling what has been described for food-grade lactic acid bacteria (LAB) producers of EPS, that are used during industrial milk fermentation (6, 7, 8, 10).

Similarly to *Streptococcus pyogenes* infection in humans, where serotype replacement in a population (21) is most likely the result of the immune status of the individuals along with the introduction of a highly virulent organism (9), the propensity of *S. iniae* to cause an invasive disease in fish is likely related not only to the immune status of the fish but also to a variety of pathogenetic mechanisms, such as the capability of the pathogen to express a different amount of capsular polysaccharide (CPS) during the various stages of the disease (25). For *S. iniae* (4, 23), as for other streptococci of medical interest, such as *S. pneumoniae* and *S. agalactiae*, the ability to cause a disease is serotype dependent; in these cases, polysaccharides are not only virulence factors but also core targets for protective immunity (2, 5, 32, 34).

New disease outbreaks among vaccinated fish point out the probability that, following selective pressure (vaccination of the entire fish population) in a closed community (such as a fish farm where millions of fish are raised contemporaneously), the pathogen has acquired novel pathogenic mechanisms. In the present work, we describe the changes that have occurred in the bacterial population, pointing out that EPS production by novel *S. iniae* clinical strains is associated with enhanced virulence, enabling the pathogen to overcome the host’s immune response and initiate infection.

**MATERIALS AND METHODS**

Bacterial strains and batch culture. All *S. iniae* strains included in this study are clinical specimens isolated from the brains of infected rainbow trout. All strains, collected for period of over 15 years, originate from the same fish farm sited in the Upper Galilee in Israel. The three ADH-positive type I strains (Dan 1, Dan 4, and Dan 15) were collected between 1987 and 1992, while the three ADH-negative type II strains (KFP 173, KFP 186, and KFP 404) were collected between 1996 and 2000. Three recent *S. iniae* strains, KFP 477, KFP 468, and KFP 523 (42), collected between 2005 and 2006, were randomly chosen from a collection of over 35 strains collected from 2005 onward. All new cultures

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† Supplemental material for this article may be found at http://aem.asm.org/.

‡ Published ahead of print on 19 September 2008.
with 2 M trifluoroacetic acid for 2 h at 100°C, reduced with NaBH4 and acetylated with acetic anhydride, as described by Albersheim et al. (1). The sugar composition of modified alditols was determined by gas chromatography (GC) with four fish), previously infected by the i.p. inoculation of 10 LD50 of S. iniae KFP 404 or KFP 477, were diluted 1:5 with PBS and subjected to EPS purification, using the methodology previously described (see “Characterization of S. iniae EPS and CPS” above). The EPS obtained from 1 ml of sera was resuspended in 200 µl of PBS; this material was used for the ELISA assay. Immulon 4 (Dynex, Chantilly, VA) plates were coated (overnight at 4°C) with 50 µl containing either EPS purified from the sera of the diseased fish or with the product obtained by the identical purification protocol applied to the sera of healthy (negative-control) fish. The plates were washed three times with PBST and blocked (with 1% PBS plus 0.1% bovine serum albumin) at RT for 60 min. The plates were then washed once with PBS, and pooled serum samples obtained from five fish from each group (1/100 to 1/3,200 final dilution in blocking buffer) were added. After 2 h of incubation at RT, the plates were washed three times with PBST and substrate (4-methylumbelliferyl phosphate) was added (50 µg/ml; 50 µl/well). Fluorescence was read on an ELISA reader (Labsystem multiscanner RC).

Statistical analysis (SAS software, version 5). The data in Fig. 1 and 2 are presented as the means ± standard deviations of the EPS concentrations of healthy and sick fish from four independent experiments performed in triplicate. The differences between the mean values were determined by a one-way analysis of variance test.

Pearson correlation coefficients were used for determining the association between the antibody levels of vaccinated fish and the protection obtained in the experimentally induced disease (Fig. 3 and 4).

In vivo production of EPS. A lecin ELISA assay, often called an enzyme-linked lectinobenton assay (ELLA) (36), based upon the modification of a procedure described elsewhere (22), was applied to detect and quantify EPS in the sera of clinically diseased fish. Sera (1 ml) from diseased fish (three pools, each with four fish), previously infected by the i.p. inoculation of 10 LD50 of S. iniae KFP 404 or KFP 477, were diluted 1:5 with PBS and subjected to EPS purification, using the methodology previously described (see “Characterization of S. iniae EPS and CPS” above). The EPS obtained from 1 ml of sera was resuspended in 200 µl of PBS; this material was used for the ELISA assay. Immulon 4 (Dynex, Chantilly, VA) plates were coated (overnight at 4°C) with 50 µl containing either EPS purified from the sera of the diseased fish or with the product obtained by the identical purification protocol applied to the sera of healthy (negative-control) fish. The plates were washed three times with PBST and blocked (with 1% PBS plus 0.1% bovine serum albumin) at RT for 60 min. The plates were then washed once with PBS, and pooled serum samples obtained from five fish from each group (1/100 to 1/3,200 final dilution in blocking buffer) were added. After 2 h of incubation at RT, the plates were washed three times with PBST and substrate (4-methylumbelliferyl phosphate) was added (50 µg/ml; 50 µl/well). Fluorescence was read on an ELISA reader (Labsystem multiscanner RC).

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The differences between the protection rates of the different vaccine preparations was analyzed by the chi-square test (Fig. 4).

**RESULTS**

*S. iniae* produces, both in vitro and in vivo, EPS that is quantitatively different from its CPS. Under fermentation conditions with pH compensation at 6.8, all recent isolates gave rise to a viscous culture, resembling the typical texture obtained during the fermentation of LAB that are EPS producers (7, 8, 10). *S. iniae* KFP 468, KFP 477, and KFP 523 yielded 323, 289, and 365 mg of EPS per liter, respectively. Under the same conditions, none of the three (ADH-positive) type I strains (Dan 1, Dan 4, and Dan 15) or the three (ADH-negative) type II strains (KFP 173, KFP 186, and KFP 404) produced visibly detectable amounts of EPS. Nevertheless, when the supernatants obtained from cultures of previous (type I and type II) strains were ethanol-precipitated (26) and analyzed by GC, it was found that these strains produced a small amount (3.9 to 5.1 μg/liter, i.e., 5 logarithmic orders less than KFP 477) of EPS.

The lectin ELISA assay analysis revealed that significant EPS production occurs also in vivo: EPS calibration and ELLA analysis of sera (Fig. 1; see also the supplemental material) demonstrate that EPS concentrations in the sera of *S. iniae* KFP 477-infected fish is 65 ng/ml, while the background (non-specific binding to the sera of healthy fish) concentration is only 5 ng/ml (P < 0.005). Quantification of the EPS level in the sera of diseased fish that were infected by the vaccine strain (*S. iniae* KFP 404) revealed that the level was 16 ng/ml, a reduction of over 75% compared to that of the KFP 477-infected fish (P < 0.01). The difference in EPS level between the negative control group and the KFP 404-infected fish was not significant (P > 0.05).

Thus, a common physiological trait, the significant production of EPS, is shared by all the recent isolates tested. EPS analysis by GC revealed the monosaccharide composition of rhamnose-fucose-ribose-arabinose-xylose-mannose-galactose-glucose at a weight ratio of 1.2:0.7:6.6:1.8:6.8:4.7:15.9 (Fig. 2). CPS monosaccharide composition was qualitatively identical to that of the EPS. However, a quantitative comparison between EPS and CPS subunits revealed that, in KFP 477 CPS, the weight ratio of the single sugars was 25.1:14.9:2.9:1.0:17.7:11.9:25.5. The CPS monosaccharide composition of the “conventional” (former) vaccine strain (KFP 404, nonproducer of EPS), composed of rhamnose-ribose-arabinose-xylose-mannose-galactose-glucose-acid, N-acetyl-d-galactosamine, and N-acetyl-d-glucosamine (23). Since North American and Israeli isolates cluster in two
distinct epidemiological clones, demonstrating the independence of the evolution of this pathogen in each of the countries (12), this finding is not surprising.

The \textit{S. iniae} EPS is the predominant immunogenic antigen. To better understand the epidemiological basis that underlies strain replacement and new outbreaks, along with the evaluation of the importance of EPS elaboration by novel strains, fish were immunized with various preparations (EPS, killed cells, or killed cells plus EPS) and challenged (for assessment of protection), and the various sera were tested for reactivity with EPS and/or killed bacteria as antigens in an ELISA assay. A former \textit{S. iniae} isolate, KFP 404, was also included. The ELISA results (Fig. 3) demonstrated that, regardless of the vaccine type—with the exception of the former isolate—all sera reacted with each of the components included in the different vaccines. However, substantial quantitative differences in recognition patterns were detected in the sera of fish immunized with various vaccine preparations based upon the novel strain (KFP 477) or its EPS. The sera obtained after vaccination with the former strain (KFP 404), differed from other sera qualitatively. Immune sera collected from the fish vaccinated with KFP 404 (former vaccine strain) hardly recognized the new KFP 477 strain or its EPS (Fig. 3A and B); poor immunologic recognition was accompanied by high mortality rates (66%) after experimental infection. These findings clearly explain the rationale for which recurrences of disease outbreaks have occurred among vaccinated fish. As shown in Fig. 4, vaccination by the new strain or its EPS resulted in significant protection rates (78% and 72% of protection, respectively), while vaccination with the previous isolate resulted in 34% protection with 92% mortality among the group of unvaccinated fish. Statistical analysis of the differences between the four vaccines revealed significantly higher protection rates among the fish vaccinated with KFP 477 killed cells, KFP 477 EPS, and KFP 477 killed cells plus EPS than KFP 404 (\(P < 0.01\), \(P < 0.002\), and \(P < 0.001\), respectively). All vaccines, including KFP 404, resulted in higher protection rates in comparison to the nonvaccinated fish (\(P < 0.01\) for KFP 404; \(P < 0.001\) for all other vaccines). Protection rates linked with the level of specific antibodies against the new isolate (OD of 1.0 and 0.8, respectively, at a dilution of 1:100), as shown in Fig. 3A (bacteria-coated ELISA plates). Correlation coefficients between the protection rates and antibody levels were 0.965 (ELISA with coated bacterial cells [Fig. 3A]) and 0.962 (ELISA with coated \textit{S. iniae} EPS [Fig. 3B]). Both coefficients are highly significant (\(P < 0.01\)). When the ELISA plates were coated with \textit{S. iniae} cells plus EPS, the correlation coefficient was determined to be 0.977 (\(P < 0.005\)). The ELISA results, besides substantiating previous results regarding serotype replacement, also point out the major role of EPS in obtaining a significant immune response. This observation is highlighted through a more particular analysis of the humoral immune response (Fig. 3B, EPS-coated ELISA plates), showing that the antibody response is directed, primarily, against the carbohydrate moieties (OD of 1.4 with the cellular vaccine or OD of 1.1 for the EPS-based vaccine). Not unexpectedly \((P < 0.001)\), the highest protection rate was that of the fish vaccinated with the new isolate (KFP 477) plus purified EPS (18% mortality), where antibody titers (Fig. 3C, bacteria plus EPS-coated ELISA plates) were the highest (OD = 1.6). The fundamental importance of anti-EPS antibodies is also revealed in the breakdown of the immune response of this group, demonstrating that the antibodies are directed against the EPS (OD = 1.3). Although, as in the case of antipneumococcal vaccines (26, 29, 35), the sole consideration of the humoral response to the \textit{S. iniae} vaccine is clearly also an oversimplification of a complex process that involves the cellular branch (13), the levels of antibody titers reliably correlated with the protection rates.

**DISCUSSION**

Evidently, the \textit{S. iniae} population structure fluctuates over time and also with geographical location. While the most dramatic change in \textit{S. iniae} population during the last decade has been the emergence of invasive type II strains able to evade the immune response elicited by vaccination with type I strains (4), present changes share some common characteristics with the former. In both instances, changes in bacterial population are associated with three main factors: (i) massive vaccination (the entire population is vaccinated routinely, which encompasses more than 3 million fish/year), (ii) closed community (all relevant farms share common water resources and are supplied by the same fry), and, as final outcome, (iii) bacterial evolution resulting in the clonal selection of strains which have gained privileged characteristics in a hostile (vaccinated) environment for the bacterium (4). Since substantial production of EPS is an attribute common only to new strains, a correlation between EPS production and the ability to evade the immune environment (where immunization was performed with the “conventional” EPS-nonproducing KFP 404 type II strain) is likely to exist.

This assumption is corroborated by the findings that vaccination with EPS alone resulted in protection rates that are largely comparable to those achieved by vaccination with killed cells (78% and 72%, respectively; \(P > 0.05\)), while protection following vaccination with a “conventional” type II strain (KFP
production and secretion of high quantities of polysaccharide by these nutrients. The role of EPS in pathogenesis has not been previously documented, although in S. agalactiae batch culture a small amount of type III CPS is not cell associated (11, 20). The phenomenon of EPS release into the medium, presumably after cell death, is notably different from EPS secretion: while CPS saccharidic polymers are assembled as CPS that are tightly associated with the cell surface, EPS saccharidic polymers are secreted into the growth medium (i.e., “slime” polysaccharides or “viscous culture”) (6). The excessive production of EPS, either as homopolysaccharides of the mutants/levans or dextran type or as a very heterogeneous group of extracellular heteropolysaccharides (7, 8, 11), has been described exclusively for food-grade LAB of industrial interest. For LAB, which live in a rich and nutritive habitat, the selective advantage of producing EPS is not evident; it was speculated (33) that EPS synthesis might be a trait that was carried over in evolution from organisms for which the polysaccharides provided a selective advantage (18, 31, 32). In this regard, S. iniae might represent the opposite event, in which a pathogen acquires the advantageous capability to produce EPS. Further studies are necessary to understand the functional role of EPS in the pathogenicity of S. iniae.

REFERENCES


404) was only 34%. This emphasizes that, similarly to what has been described for several streptococcal species of medical interest (including S. agalactiae [31] and S. suis [40]), enterococci (19), and pneumococci (2, 3, 14, 32), as well as for S. iniae itself (23, 25), polysaccharides are not only a major virulence factor but also core targets for protective immunity and that protection is related to levels of anti-polysaccharide antibodies present in serum. For S. iniae immunity, the role of EPS and anti-EPS antibodies is divulged in Fig. 3A and B, which show that anti-EPS antibodies produced by fish immunized by EPS alone (OD = 0.8; 72% protection) recognize whole cells to an extent that approximates that of the anti-EPS antibodies produced after vaccination with whole cells (OD = 1.0) and that the difference in the protection degree of the former and latter fish (78%) is statistically insignificant (P > 0.05). One might assume that, since protection following vaccination with EPS alone is not full (72%) despite the elevated titers of anti-EPS antibodies, the role of these anti-EPS antibodies should be reassessed. Since fish are genetically heterogeneous, and since vaccination with preparations that included whole cells did not improve protection significantly (78% and 82% protection for whole cells and cells plus EPS, respectively), it is reasonable to hypothesize that the “low responders” failed in producing sufficient levels of anti-EPS antibodies and succumbed in the challenge assays. The fact that immunization by KFP 477 without EPS resulted in significant protection does not contrast with the role of EPS, as before being released into the medium, this molecule is cell-associated; it is logical that the KFP 477 whole cells were coated by still-unreleased EPS and that the slow degradation of the bacteria enhanced the immune response toward EPS (depo effect). This assumption is strengthened by the ELISA results, showing that titers of the anti-EPS antibodies following vaccination with KFP 477 cells (OD = 1.5) were still higher than the anti-EPS titers following vaccination by EPS alone (OD = 1.18) (Fig. 4B). Nonetheless, the considerable degree of protection attained by immunization with EPS alone does not denote that other antigens, such as the S. iniae M-like protein, are potentially of central importance (24). Even for Streptococcus pneumoniae, for which the current vaccine is based upon CPS (18, 30), cell-wall-associated proteins have been shown to confer considerable immunity in laboratory models and are now being explored as future vaccine candidates (16, 37).

Contrary to eradication, vaccination merely diminishes the pathogen’s prevalence, enabling a natural selection to take place. Therefore, it is not surprising that in a closed community with constant selective pressure (as a fish farm practicing vaccination of the entire stock), the adaptation of a pathogen through clonal selection of virulent strains that have appropriated novel antigenic/virulence factors has occurred. A similar event has also been described for S. pyogenes, where serotype replacement in a population (semiclosed community) was most likely the result of the immune status of the individuals along with the introduction of a highly virulent organism (9, 21). Increases in carriage of nonvaccine serotypes and serotype replacement by strains that are not represented in the S. pneumoniae vaccine were also divulged (14, 27). However, the finding that the mechanism of S. iniae adaptation resides in the production and secretion of high quantities of polysaccharide (over 365 mg) was unexpected. To the best of our knowledge, EPS production in pathogenic streptococci has not been previously documented, although in S. agalactiae batch culture a small amount of type III CPS is not cell associated (11, 20). The phenomenon of CPS release into the medium, presumably after cell death, is notably different from EPS secretion: while CPS saccharidic polymers are assembled as CPS that are tightly associated with the cell surface, EPS saccharidic polymers are secreted into the growth medium (i.e., “slime” polysaccharides or “viscous culture”) (6). The excessive production of EPS, either as homopolysaccharides of the mutants/levans or dextran type or as a very heterogeneous group of extracellular heteropolysaccharides (7, 8, 11), has been described exclusively for food-grade LAB of industrial interest. For LAB, which live in a rich and nutritive habitat, the selective advantage of producing EPS is not evident; it was speculated (33) that EPS synthesis might be a trait that was carried over in evolution from organisms for which the polysaccharides provided a selective advantage (18, 31, 32). In this regard, S. iniae might represent the opposite event, in which a pathogen acquires the advantageous capability to produce EPS. Further studies are necessary to understand the functional role of EPS in the pathogenicity of S. iniae.


