

Studies on the Serotypes and Genetic Structure of Capsular Polysaccharide Biosynthesis Loci of the Fish Pathogen *Streptococcus parauberis*

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Streptococcus parauberis, a Gram-positive coccus, was reported as a fish pathogen for the first time in Spain in 1996. In Japan, *S. parauberis* has been recognized as a pathogen of Japanese flounder *Paralichthys olivaceus* since 2002. According to the results of slide agglutination test with rabbit antisera, the isolates from flounder have been divided into two serotypes, termed serotypes I and II. In streptococci, a variety of serotypes are generally due to differences of the capsular polysaccharide (CPS) antigens on their cell-surface, which are synthesized by the *cps* gene cluster on the chromosome. Since 2008, *S. parauberis* isolates that did not agglutinate with anti-serotype I or II sera were occasionally found, suspecting that there exist new serotypes in *S. parauberis*. Accurate identification of this pathogen is important for the epidemiological study, pathogenesis study, and therapy of the infection. This study was aimed to reexamine the serological relationship among the stocked *S. parauberis* strains including the non-agglutinating ones and also to investigate the correlation between the serotypes and structure of *cps* gene clusters of *S. parauberis*.

In Chapter 1, the serotypic position of stocked *S. parauberis* strains was reexamined using a newly prepared rabbit antiserum against a non-agglutinating strain (NUF1071) as well as previously prepared anti-serotype I and II sera, and also pulsed-field gel electrophoresis (PFGE) was conducted to clarify the genetic relationship among the strains. An antiserum cross-absorption test and microtiter agglutination test revealed that the serotype I was divided into three subserotypes, designated Ia, Ib and Ic, of which the non-agglutinating strains belonged to the subserotype Ic. Of the 104 serotype I strains, 6, 91 and 7 strains belonged to subserotypes Ia, Ib and Ic, respectively. Formalin-killed cells (FKC) of subserotype Ia and Ic strains showed high agglutination titers against the anti-Ia, so far being used as an anti-serotype I serum, and Ic sera, respectively. Subserotype Ib strains agglutinated with both sera. In PFGE analysis, the stocked 188 *S. parauberis* strains were classified into three clusters; clusters I, II and III consisted of subserotypes Ib/Ic, Ia and II, respectively. Non-typeable (NT) strains which did not agglutinate with both anti-serotype I and II sera were thought to be non-capsulated and belonged to Cluster I or III. Identification of pathogens with both serotyping and genotyping is more useful for epidemiological study

In Chapter 2, using inverse-PCR technique, the nucleotide sequences of the *cps* loci of six

representative strains were determined. The length of the *cps* loci of *S. parauberis* KRS02083 (Ia), NUF1003 (Ib), NUF1071 (Ic), NUF1032 (II), 2007-1 (NT, Cluster I) and NUF1095 (NT, Cluster III) was 18,910, 19,334, 19,334, 19,920, 19,334 and 19,920 bp, respectively. The genes involved in CPS biosynthesis comprised a gene cluster with cassette-like structure, and type-specific genes were flanked by conserved genes. The upper conserved region of the *cps* locus consisted of five regulatory genes and one processing gene, which were common to all the six strains. The type-specific region contained the genes that encoded an initial glycosyltransferase, polysaccharide polymerase (*wzy*), flippase (*wzx*), glycosyltransferases, acetyltransferases and aminotransferases and modifying enzymes. These structures suggest that the capsule of *S. parauberis* is synthesized by the Wzy-dependent pathway. Although the type-specific region was varied among subserotypes Ia and Ib and serotype II, several common genes existed between subserotypes Ia and Ib. This would explain the cross reactivity in agglutination test between subserotypes Ia and Ib. The type-specific regions of subserotype Ic/NT (Cluster I) and NT (Cluster III) had the same genetic structure as subserotype Ib and serotype II respectively, there were only a few base substitutions found between them. Three of seven subserotype Ic strains harbored an IS element in the *cps* locus. This and a few base substitutions in the genes of *cps* locus would cause the change of amino acid and protein structure which resulted in the loss of enzyme activity and, hence, caused the diversification of capsular polysaccharide or non-capsulation .

In Chapter 3, a multiplex PCR method for the detection of *S. parauberis* serotypes was developed base on the studies in the previous chapter. Serotype-specific primer sets were designed from the *wzy* gene sequences of *S. parauberis* with the expected product length of 213, 303 and 413 bp for subserotypes Ia and Ib/Ic and serotype II, respectively. The test results were consistent with those of agglutination test using antisera. Moreover, NT strains could be classified into serotype Ib/Ic or II which the result was consistent with PFGE. None of the other streptococcal species or the other pathogens of Japanese flounder showed positive reaction. Accordingly, the present multiplex PCR method was considered as an alternative of agglutination test for identification of *S. parauberis* and determination of the serotypes simultaneously.

In summary, the serological investigation revealed that the isolates of *S. parauberis* from Japanese flounder were divided into five serological phenotypes (subserotypes Ia, Ib and Ic, serotype II and nontypeable). Genotyping by pulsed-field gel electrophoresis can be a good supplementary for traditional serotyping of *S. parauberis*. The serological phenotypes were associated with the structure of *cps* locus, and the study on the *cps* locus may lead a better view to understand the mechanism of arising serological diversity. The multiplex PCR developed base on the *wzy* gene sequences would lead to a rapid and precise diagnosis of *S. parauberis* and also determination of serotypes simultaneously.