Cloning of phosphoglucomutase gene (pgm) in Streptococcus parauberis

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Here, we have cloned and sequenced *Streptococcus parauberis pgm* gene, encoding the enzyme phosphoglucomutase (PGM), which is known to be in association with virulence in other streptococcal species. The PGM of *S. parauberis* is the most closely related to that of *S. iniae* based on their amino acid sequences.

Key words : Streptococcus parauberis, pgm gene, phosphoglucomutase, virulence

Streptococcicosis is one of major problems in wild and farmed populations of diverse freshwater and marine fishes worldwide (Austin and Austin, 2007). The main pathogenic species responsible for fish streptococcicosis, which have been reported in many different countries are Streptococcus iniae, Streptococcus parauberis. Streptococcus difficilis, Vagococcus salmoninarum, Lactococcus piscium and Lactococcus garviae (Domeénech et al., 1996; Eldar et al., 1994; Eldar et al., 1996; Zlotkin et al., 1998). In particular, S. iniae and S. parauberis were initially thought to be dominant causative agents in streptococcal diseases of olive flounder (Paralichthys olivaceus), a major aquacultured species in Korea. However, S. parauberis tends to be as often isolated as S. iniae from diseased olive flounder in recent years (Cho et al., 2007; Jeong et al., 2006). Indeed, initial disease outbreaks caused by S. parauberis occurred in Spanish turbot farms between 1993 and 1996 (Toranzo *et al.*, 1994; Domeénech *et al.*, 1996). Although a vaccine has been available for the disease, Streptococcicosis continues to be endemic in turbot farms (Curras *et al.*, 2002). Similarly, streptococcal vaccines available in the Korean market did not seem to confer olive flounder protection against *S. parauberis* infection. Althougn infection route of *S. parauberis* is known to be horizontal through the water or direct contact with infected fish (Romalde *et al.*, 1996), virulence factors of this pathogen are still largely unknown. Thus, understanding of pathogenicity of *S. parauberis* is an urgent necessity and it will provide important information for the development of an effective prophylaxis.

The enzyme phosphoglucomutase (PGM) is responsible for the conversion of D-glucose 1-phosphate into D-glucose 6-phosphate, and it participates in both the breakdown and synthesis of glucose. Also, it is known to play an important role in polysaccharide capsule production and pathogenicity in a variety of gram-negative

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and gram-positive bacterial pathogens (Buchanan *et al.*, 2005). Phosphoglucomutase gene (*pgm*) of varying streptococci, including *S. iniae* (Buchanan *et al.*, 2005), *S. gordonii* (Bizzini *et al.*, 2007) and *S. pneumonia* (Hardy *et al.*, 2001), has been identified and characterized, suggesting that the PGM plays a critical role in virulence. In particular, the PGM of *S. iniae* is associated with normal cell wall morphology, surface capsule expression, and resistance to innate immune clearance mechanism (Buchanan *et al.*, 2005). Here, we describe the nucleotide and deduced amino acid sequence of the *pgm* of *S. parauberis*, and compare with those of other streptococci.

Materials and Methods

S. parauberis JJI51, isolated from the kidney of diseased olive flounder in 2005, was used for the pgm cloning. The bacterial strain, identified as S. parauberis based on its 16S rRNA gene sequence (GenBank accession number FJ009631), was routinely cultured on Todd-Heweitt agar or in broth at 27° C.

A degenerate primer set for *pgm* (pgmF and pgmR) (Table 1) was designed using conserved regions of the published nucleotide sequences of *S. pyogenes* (Accession number NC_002737), *S. mutans* (Accession number NC_004350) and *S. iniae* (Accession number AY846302). The nucleotide sequence of the complete *pgm* was obtained using the LA PCR in vitro cloning kit (TaKaRa Bio Inc., Otsu, Japan). Primers, pgm5I (inverse primer)/pgm5IN (inverse nested primer) and pgm3I/pgm3IN, were used to amplify the 5' and the

3' end of the pgm, respectively. Chromosomal DNA of S. parauberis, used as template DNA, was digested with restriction enzymes, EcoRI and PstI, and the DNA fragments were ligated into the corresponding cassettes of the LA PCR in vitro cloning kit. The ligated cassettes were used for the 1st PCR using C1 (provided by the manufacturer) and a pgm3I or a pgm5I primer. After 10 min of initial denaturation at 94 $^{\circ}$ C, the following conditions were applied; 30 cycles of 30 s at 94° C, 30 s at 55 °C and 4 min at 72 °C, and 7 min of final extension. The 1st PCR products were used for 2nd PCR using C2 (provided by the manufacturer) and pgm3IN or pgm5IN with the same PCR condition, except for an extension step that was for 2 min at 72°C. Amplification products were analysed by electrophoresis in 1% (w/v) agarose gel containing ethidium bromide (1 ng/ml). The PCR products, purified with a OIAquick PCR purification kit (Oiagen, Germany) as described by the manufacturer's instructions, were cloned by using Escherichia coli DH5a and pGEM-T easy vector (Promega, Madison, WI, USA). Sequencing reactions were carried out using the BigDye terminator v.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), and sequencing analysis was performed using a Prism 310 Genetic Analyzer (Applied Biosystems). DNA sequence identities and homology of amino acid sequence was analyzed by BioEdit Ver. 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The multiple alignment of protein sequences was produced by the Clustal W program

(http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html).

Primer	Orientation of sequence	Base sequences (5'-3')
pgmF	Forward	ATGACTTATACAGAAAATTATC
pgmR	Reverse	AGAA(C/T)TTGATTTT(C/T)GG(C/T)TC(G/A/T)G
pgm5I	Reverse	TCAAAGGCAAATTCTGGTGA
pgm5IN	Reverse	TCCTGCACCAATGTAACCAC
pgm3I	Forward	TGCTGAAAGCTACAGTGCAAC
pgm3IN	Forward	GTGATAAAGATGCTATTCAAGCCAG

Table 1. PCR primers used for the identification of the pgm gene of Streptococcus parauberis

Results and Discussion

The nucleotide sequence for S. parauberis pgm identified in this study has been deposited in GenBank under accession number FJ004970. Multiple alignment of pgm (Streptococcus parauberis) deduced amino acid sequence with other pgm proteins is shown in Fig. 1. DNA sequence identities between pgm of S. parauberis and those of other streptococcal species are as follows; S. iniae (AY846302, 79.1%), S. mutans (NC 004350, 77.5%), and S. pyogenes (NC_002737, 77.3%). Homology of amino acid sequence of the PGM between four streptococcal species (S. parauberis, S. iniae, S. pyogenes, and S. mutans) is shown in Table 2. Of these, the PGM of S. parauberis is the most closely related to that of S. iniae. Primary structure of the putative pgm of S. parauberis was analyzed by a SMART architecture research computer program

(http://smart.EMBL-heidelberg.de/) (Schultz *et al.*, 2000). The gene of *S. parauberis* consists of four domains with c-terminal in its 4th one, which is different from the other streptococcal species composed of only three main domains (I, II and III). It is inferred that domain I, II, III and IV of *S. parauberis* contain the catalytic phosphoserine residue, a metal-binding loop, the sugar-binding loop, and a phosphate-binding site, respectively (Shackelford *et al.*, 2004).

The *pgm* gene of several streptococcal species has been sequenced, some of which show that the gene is in association with virulence. Indeed, a PGM mutant strain of *S. iniae* may have value as a live vaccine as the mutant was able to stimulate an immune response (Buchanan *et al.*, 2005). In line with this, *pgm* of *S. parauberis* may have an important role in virulence mechanism. Further study will be required to determine the functional role of the gene.

Table 2. Degree of homology of Streptococcus parauberis PGM to other streptococcal PGMs (amino acid sequences)

Destavial anasias	vs. S. parauberis PGM	
Bacterial species —	% identity	% similarity
S. iniae (AY846302)	88.5	92.8
S. pyogenes (NP_664668)	85.5	92.8
S. agalactiae (ZP_00785392)	85.1	92.1
S. mutans (NP_721469)	84.1	90.4

	<u>1</u> →
S. parauberis	MTY TENYQKWLAVST LPDYIMEELKSMDDK TME DAFYTN LEFGTAGMRGYI GAG TNR INI YVVRQA TEGLAKIVE SKGQD 80
S. iniae	MTY TENYQKWINASNIPDYIK DELLAMDEKTKE DAFYTNLEFGTAGMRGYI GAG TNR INVFVVRQA TEGLAKIVE SKGQA 80
S. pyogenes	MTY TEN FOKWLD FEOLPD YLROELLSMDEKTKE DAFYINLEFGTAGMRGYIGAGTNRINIYVVROA TEGLAKLIETKGEE 80
S. agalactiae	MTY TEN FOKWLD FEOLPD YLR OELLSMDEKTKE DAF YTNLEF GTAGMRGYI GAG TNR INI YVVROA TEG LAKLIE TKGEE 80
S. mutans	MVY QENYQKWLA YAKLPDYLHQELLAMDEKTKE DAFYINLEFGTAGMRGYIGAGTNRINIYVVRQA TEGLAQLIETKGDD 80
Clustal Consensus	*.* **:**** ***** :** :**:*************
	P-Ser
S. parauberis	AKDAGIAIAYDSRHFSPEFAFESAQVLAAHGIKSYVFESLRPTPELSFAVRHLGAFAGIMITASHNPAPFNGYKVYGADG 160
S. iniae	AKDAGVAIAYDSRHFSPEFAFESAQVLAAHGIKSYVFESLRPTPELSFAVRHLGAVAGIMVTASHNPAPFNGYKVYGADG 160
S. pyogenes	AKKRGVAIAYDSRHFSPEFAFESAQVLAQHGIKSYVFEALRPTPELSFAVRHLNAYAGIMVTASHNPAPFNGYKVYGQDG 160
S. agalactiae	AKKRGVAIAYDSRHFSPEFAFESAQVLAQHGIKSYVFETLRPTPELSFAVRHLNAYAGIMVTASHNPAPFNGYKVYGQDG 160
S. mutans	VKKRGVAIAYDSRHFSQEFAFESAQVLAKHGIKAYVFESLRPTPELSFTVRHLGTFAGIMVTASHNPAPFNGYKVYGEDG 160
Clustal Consensus	142
	II>
S. parauberis	GOMPPVDADALTDYIRAIEDPF\$IQLADLEEAKETGLIEVIGEAIDKEYLKEVKDVNINQDLIDNYGRDMKIVYTPLHGT 240
S. iniae	GQMPPADADALTDYIRAIEDPF\$IVLADLEDSKSNGLIEVIGEAIDTEYLKEVKDVTINQELIDTFGRDMKIVYTPLHGT 240
S. pyogenes	GQL PPA DADALT DFI RAI ENPFAVELADLDESK SSGLIQVIGEDVDIE YLREVKDVN INQDLINNFGKDMKI VYT PLHGT 240
S. agalactiae	GOLPPADADALTDFIRAIENPFAVELADLDESKSAGLIQVIGEDVDMEYLREVKDVNINQDLINNFGKDMKIVYTPLHGT 240
S. mutans	GOM PPF DADALT DFIRAINDPF SIELADLE ESKASGLIE VIGEAVDID YLKEVKDVNINOKLI DEYGKDMKI VYT PLHGT 240
Clustal Consensus	
	Metal-binding loop
S. parauberis	GEMLAR RALAQA GFE SVE VVE AQA KPD PDF STVKSPNPE SQAAFA LAE ELG RQVNAD VLVATD PDA DRLGVE IRQEDG SY 320
S. iniae	GEMLTR RALAQAGFE SVEVVE SQAKAD PNFSTVASPNPE SQEAFALAE ELGRTVNAD VLVATD PDADRLGVE IRQADGSY 320
S. pyogenes	GEMLITRRALAQAGFE SVVVVE SQAKAD PDF STVKSPNPE SQAAFA LAE ELGREVNADVLVATD PDADRLGVE IRQPDGSY 320
S. agalactiae	GEMLTR RALAQAGFE SVVVVE SQAKAD PDF STVKSPNPE SQAAFALAE ELGREVDAD VLVATD PDADRLGVE IRQ PDG SY 320
S. mutans	GEMLARRALAQAGED SVQVVEAQAVPD PDF STVKSPNPENQE AFR LAE ELGRQVDADVLVATD PDA DRLGVE IRQADGSY 320
Clustal Consensus	
	III→ WNLSGNQIGALIAKYILEAHKOAGTLPENAALAKSIVSTELVTKIAESYSATMFNVLTGFKFIAEKIQEFEEKHNYTYMF 400
S. parauberis	
S. iniae	WNLSGNQIGALIAKY ILEAHKVAG TLPVNAALAKSIVSTELVTKIAESYGATMFNXLTGFKFIAEKIQE FEETHNHTYMF 400 KNLSGNOIGALIAKY ILEAHKTAG TLPENAALAKSIVSTELVTKIAESYGATMFNVLTGFKFIAEKIOE FEEKHNHTYMF
S. pyogenes	KNLSGNQIGAIIAKYILEAHKTAGTLPENAALAKSIVSTELVTKIAESYGATMFNVLTGFKFIAEKIQEFEEKHNHYMF KNLSGNQIGAIIAKYILEAHKTAGTLPENAALAKSIVSTELVTKIAESYGATMFNVLTGFKFIAEKIQEFEEKHNHYMF 400
S. agalactiae	
S. mutans Clustal Consensus	KNLSGNQIGAIIAKYILEAHKTAGSLPTNAALCKSIVSTELVSKIAESYGATMFNVLTGFKFIAEKIQEFEEKHNHTYMF 400 357
Clustal Consensus	
S. parauberis	Sugar-binding loop IV-> GFEESFGYLIKPFVRDKDAIQAVLIVAEIAAYYRSRGLTLADGIDEIKKEYGYFAEKTISLTLSGVDGAAQINLIMNKFR 480
S. iniae	GFEESFGYLIKPFVRDKDAIQAVLIVAEIAAYYRSKGLIADGIDDIKKEYGYFAEKTISLILSGVDGASQIKAIMDKFR 480
S. pyogenes	GFEESFGYLIKPFVRDKDAIQAVLIVAEIAAYYRSRGIILADGIDEIKKEYGYFAEKTISVILSGYDGAAEIKKIMDKFR 480
S. galactiae	GFELST GYLIKP FYRDKDAIQAVLIVAEIAAYYRSRGIILADGIDEIK KEYGYFAEKTISVILSGYDGAAEIKKIMNKER 480
S. mutans	GFESSFGYLIKFFVRDKDALQAVLIVAEIAAYPRSGLILADGIDEIKKEYGYFAEKISVILSGVDGATEIKKIMDKFR 480
Clustal Consensus	GEDEGELERPEVRICATIONEDIATION DE LA CONTRACTION DE LA CONTRACTIONE DE L
Clustal Consensus	966
	Descripto hinding loop
S. parauberis	Phosphate-binding loop DNAPSQFNNTEIILSEDFQEQTAKDAQGNVKPLTTPPSNVLKYTLADDSWFAVRPSGTEPKIKFYIATVGETLEKAEEKI 560
S. iniae	OSAPTS FNOTE I VLSEDFLEOTAK SADG-T SPLTTP PSNVLKYTLADD SWFAVR PSGTEPKIKFYVATVGOT LLEAEFKI 559
S. pyogenes	ENGPKOFNNTDIVLLEDFOKOIATKNDGTISNLTIPPSNVLKYTLADDSWIAVRPSGTEPKIKFYIATVGNDLADAETKI 560
S. agalactiae	ENGPROPRINTDIVILEDFORQTATINDGTISH.ITPPSNVLKYTLADDSWIAVRPSGTEPKIKFYIATIGDTLDIAQEKI 560
S. mutans	GDA PKOENA TDVVKLEDFQAQTAT TADG-IEKLTTPPSNVLKYILSDA SWI AVRPGTEPKIKFYI ATVQNNSEDAQVKI 559
Clustal Consensus	Guardina primer de la cale a cale
CLUS DEL CONSENSUS	
S. parauberis	ATLEKEINAFVD 572
S. inize	KTIEREINTFVD 571
S. pyogenes	ANIEKEITTFVN 572
S. agalactiae	ANIETE INTFVG 572
S. mutans	ANIEREINDFVG 571
Clustal Consensus	

Fig. 1. Multiple alignment of *pgm* (*Streptococcus parauberis*) amino acid sequence with other *pgm* proteins. The putative conserved functional domains of *pgm* of *S. parauberis* are boxed in gray, which are indicated by I, II, III and IV. The residues identical in all sequences are shown with asterisks (*), whereas those with strong homologies and weak similarities are marked by colons (:) and dots (.), respectively. The accession numbers of *pgm* sequences are listed in Table 2.

Acknowledgements

References

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Bizzini, A., Majcherczyk, P., Beggah-Moller, S., Soldo, B.,

Entenza, J.M., Gaillard, M., Moreillon, P. and Lazarevic, V.: Effects of α-phosphoglucomutase deficiency on cell wall properties and fitness in *Streptococcus gordonii*. Microbiol., 153:490-498, 2007.

- Buchanan, J.T., Stannard, J.A., Lauth, X., Ostland, V.E., Powell, H.C., Westerman, M.E. and Nizet, V.: *Streptococcus iniae* phosphoglucomutase is a virulence factor and a target for vaccine development. Infect. Immun., 73:6935-6944, 2005.
- Cho, M.Y., Oh, Y.K., Lee, D.C., Kim, J.H. and Park, M.A.: Geographical comparison on different methods for identification of *Streptococcus parauberis* isolated from cultured olive flounder, *Paralichthys olivaceus*, J. Fish Pathol., 20:49-60, 2007.
- Curras, M., Magarinos, B., Toranzo, A.E. and Romalde, J.L.: Dormancy as a survival strategy of the fish pathogen *Streptococcus parauberis* in the marine environment. Dis. Aquat. Org., 52:129-136, 2003.
- Domeénech, A., Derenaáandez-Garayzábal, J.F., Pascual, C., García, J.A., Cutuli, M.T., Moreno, M.A., Collins, M.D. and Dominguez, L.: Streptococcosis in cultured turbot, *Scophthalmus maximus* (L.), associated with *Streptococcus parauberis*. J. Fish Dis., 19:33-38, 1996.
- Eldar, A., Bejerano, Y. and Bercovier, H.: Streptococcus shiloi and Streptococcus difficile: two new streptococcal species causing a meningoencephalitis in fish. Curr. Microbiol., 28:139-143, 1994.
- Eldar, A., Ghittino, C., Asanta, L., Bozzetta, E., Goria, M., Prearo, M. and Bercovier, H.: *Enterococcus* seriolicida is a junior synonym of *Lactococcus* garvieae, a causative agent of septicemia and

meningoencephalitis in fish. Curr. Microbiol., 32:85-88, 1996.

- Hardy, G.G., Magee, A.D., Ventura, C.L., Caimano, M.J. and Yother, J.: Essential role for cellular phosphoglucomutase in virulence of type 3 *Streptococcus pneumonia*. Infect. immun., 69: 2309-2317, 2001.
- Jeong, Y.U., Kang, C.Y., Kim, M.J., Heo, M.S., Oh, D.C. and Kang, B.J.: Characterization of Streptococcosis occurrence and molecular identification of the pathogens of cultured flounder in Jeju island. J. Korean Soc. Microbiol., 42:199-204, 2006.
- Romalde, J.L., Magariños, B., Nuñez, S., Barja, J.L. and Toranzo, A.E.: Host range susceptibility of *Enterococcus* sp. strains isolated from diseased turbot: possible routes of infection. Appl. Environ. Microbiol., 62, 607-611, 1996.
- Schultz, J., Copley, R.R., Doerks, T., Ponting, C.P. and Bork, P.: SMART: a web-based tool for the study of genetically mobile domains. Nucleic Acids Res., 28:231-234, 2000.
- Shackelford, G.S., Regni, C.A. and Beamer, L.J.: Evolutionary trace analysis of the D-phosphohexomutase superfamily. Protein Sci., 13:2130-2138, 2004.
- Toranzo, A.E., Devesa, S., Heinen, P., Riaza, A., Nuñez, S. and Barja, J.L.: Streptococcosis in cultured turbot caused by an *Enterococcus*-like bacterium. Bull. Eur. Assoc. Fish Pathol., 14:19-23, 1994.
- Zlotkin, A., Hershko, H. and Eldar, A.: Possible transmission of *Streptococcus iniae* from wild fish to cultured marine fish. Appl. Environ. Microbiol., 64: 4065-4067, 1998.

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