Studies on the pathogenic mechanisms of *Streptococcus intermedius*

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TABLE OF CONTENTS

2. LacR mutations are responsible for increased intermedilysin production and virulence

CHAPTER Ⅰ

General introduction

Streptococcus intermedius is a facultatively anaerobic member of the normal flora of the human oral cavity and the upper respiratory, gastrointestinal, and female urogenital tracts. This pathogen belongs to the Anginosus group of streptococci (AGS), which also includes *Streptococcus anginosus* and *Streptococcus constellatus* [Whiley *et al*., 1990, 1992]. Taxonomical studies show that *Streptococcus anginosus* consists of 2 subspecies: subsp. *anginosus* and subsp. *whileyi*. *Streptococcus constellatus* consists of 3 subspecies: subsp. *constellatus*, subsp. *pharyngis*, and subsp. *viborgensis* [Jensen *et al*., 2013]. Members of AGS tend to form local suppurative infections, and these organisms are the most common pathogens associated with bacterial intracerebral abscesses [Claridge *et al*., 2001, Jacobs *et al*., 1995, Jerng *et al*., 1997, Ruoff 1988, Whiley *et al*., 1992]. *S. intermedius* is an important human pathogen and a leading cause of deep-seated infections, such as in brain and liver abscesses [Whiley *et al*., 1990, 1992]. Among AGS species, only *S. intermedius* has the *ily* gene that encodes a cytolysin called intermedilysin (ILY), which is a member of the cholesterol-dependent cytolysin (CDC) family. In contrast to other CDC family members, ILY can bind specifically to the glycosylphosphatidylinositol-linked membrane protein human CD59: a regulator of the terminal pathway of complement action in humans [Giddings *et al*., 2004]. ILY is beleved to be the major virulence factor of *S. intermedius*, essential for invasion of and cytotoxicity to human cells, due to the following: (i) the production level of ILY from isolates found in deep-seated abscesses is 6.2- to 10.2-fold higher than that from the strains found in normal habitats, such as dental plaque, in contrast to the expression levels of other potential virulence factors, such as hyaluronidase and sialidase, where no significant difference in levels has been

found [Nagamune *et al.*, 2000] ; (ii) an *ily* knockout strain showed greatly decreased adherence, invasion, and cytotoxicity of human liver (HepG2) cells [Sukeno *et al*., 2005]. Therefore, investigation of the mechanisms that regulate *ily* expression could help elucidate how *S. intermedius* mediates its pathogenicity by controlling the amount of ILY secreted. To date three factors have been reported to control the expression of *ily*. The first is autoinducer 2 (AI-2) (a LuxS product used by several bacteria in quorum-sensing signaling), which is reported to be an exponential growth phase-specific activator of *ily* transcription [Pecharki *et al.*, 2008]. Subsequently we could reveal that a catabolite control protein A (CcpA) and the lactose phosphotransferase system repressor (LacR) regulate transcription of *ily* by binding to its promoter region [Tomoyasu *et al.*, 2010, 2013]. These results strongly suggest that the amount and the type of sugar in surrounding of the bacterial cell are important factors in the pathogenicity of *S. intermedius*. Therefore, we searched for the glycosidase gene(s) in the *S. intermedius* chromosome and discovered a novel Multi-substrate glycosidase A, "MsgA" that has β-D-galactosidase, β-D-fucosidase, *N*-acetyl-β-D-glucosaminidase, and *N*-acetyl-β-D-galactosaminidase activities [Imaki *et al*., in press]. The author showed detail of the results of our research in CHAPTER Ⅱ.

CHAPTER Ⅱ

1. Role of Catabolite Control Protein A in the Regulation of Intermedilysin Production

1 – 1. Introduction

Genome-wide studies such as signature-tagged mutagenesis screens have identified the genes involved in basic metabolic processes, including the catabolism of complex carbohydrates, as crucial to the pathogenesis of disease caused by many streptococci [Shelburne *et al.*, 2008a, b]. In addition, the numerous genes encoding proteins with known or putative carbohydrate metabolism functions are up-regulated during Group A streptococci (GAS, *Streptococcus pyogenes*) and *Streptococcus pneumoniae* infections [Graham *et al*., 2006, Orihuela *et al*., 2004]. Several recent reports have indicated that CcpA regulates not only the genes for catabolism but also some genes for virulence factors including streptolysin S of GAS [Kinkel & McIver. 2008, Shelburne *et al*., 2008b]. Therefore, carbohydrate catabolism genes mediating transcriptional control by CcpA-dependent carbon catabolite repression (CCR) are thought to have an important function in regulating the pathogenicity of streptococci. The mechanism of CCR in low-GC-content Gram-positive bacteria has been clarified by studies on *Bacillus subtilis* [Deutscher. 2008, Fujita. 2009, Görke & Stülke. 2008, Warner & Lolkema. 2003]. CCR is controlled by the histidine-containing protein (HPr), phosphoenolpyruvate:sugar phosphotransferase system protein, and CcpA. The uptake of a preferred carbohydrate such as glucose, fructose, or mannose leads to an increase in cellular levels of fructose-1,6-bisphosphate, which triggers ATP-dependent HPr kinase/phosphatase-catalyzed phosphorylation of HPr at Ser-46. Only seryl-phosphorylated forms of HPr can bind to CcpA, and the P-Ser-HPr/CcpA complexes bind in turn to the

catabolite-repressible element (*cre*). If the *cre* site is located within the promoter region or open reading frame, CcpA binding inhibits the RNA polymerase interaction with the promoter or its progression through to DNA, thereby repressing transcription [Kim *et al*., 2005].

To determine the expression control mechanisms of *ily*, we analyzed the nucleotide sequence of the *ily* promoter region and found a highly homologous region to the *cre* consensus sequence, WWTGNAARCGNWWWCAWW (where W = A or T, N = any base, and R = G or A), with alignment of 22 *cres* functional in *B. subtilis* [Miwa *et al*., 2000]. In addition, we introduced a *ccpA* knockout mutation (Δ*ccpA*) and *cre* mutation into UNS38 that is ILY-high producing strain isolated from human brain abscess to investigate whether *ily* expression is controlled by CcpA-dependent CCR.

1 – 2. Materials and Methods

1 – 2 – 1. Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. *Streptococcus intermedius* was cultured at 37°C under anaerobic conditions (CO_2:H_2:N_2, 5:10:85). Brain heart infusion (BHI) broth (Becton-Dickinson, Palo Alto, CA), BHI broth without dextrose (United States Biological, Swampscott, MA) supplemented with glucose at the concentrations indicated, or 3-(*N*-morpholino)propanesulfonic acid (MOPS)-buffered BHI (MOPS-BHI) medium was used for culture. The MOPS-BHI medium contained 100 mM MOPS buffer (pH 7.4) and 18.5 g/liter BHI broth or 17.5 g/liter BHI broth without dextrose, and it was supplemented with glucose or other sugars at the specified concentrations.

Escherichia coli was grown in Luria-Bertani (LB) medium at 37°C under aerobic conditions. Antibiotics were added at the following concentrations: ampicillin at 50 μg/ml for *E. coli* culture, chloramphenicol (Cm) at 20 μg/ml for *E. coli* and 2 μg/ml for *S. intermedius*, and erythromycin (Em) at 1 μg/ml for *S. intermedius*.

1 – 2 – 2. Databases and multiple sequence alignment.

Nucleotide and protein sequences were obtained from GenBank or the Microbes genomic BLAST databases by an Entrez cross-database search at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/gquery). A multiple sequence alignment was constructed with the Parallel PRRN program at the Kyoto University Bioinformatics Center, Japan (http://www.genome.jp/tools/prrn/).

Table 1: Bacterial strains and plasmids used in this study

Strains	Relevant characteristics	Reference or source
S. intermedius		
UNS38	ILY-high producing strain from human brain abscess	Sukeno *et al.*, 2005
UNS38 B3	*ily* knockout strain derived from UNS38	Sukeno *et al.*, 2005
UNS38 Δ*ccpA*	*ccpA* knockout strain derived from strain UNS38	This study
UNS38 EM^r	Insertion of EM cassette in front of *ily* promoter region of UNS38	This study
UNS38 *creE*	Same as UNS38 but introduced 3 point mutations in the *cre* site	This study
UNS38 *creS*	Same as UNS38 but introduced 2 nucleotide deletions and 1 point mutation in a *cre* site	This study
E. coli		
C600	*thr-1 leuB6 thi-1 lacY supE44 rfbD1 fhuA21*	Lab. collection
DH5Zα1	F^- *endA1 hsdR17*($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ*(lacZYA-argF)*U169 *deoR* φ80d*lacZ*ΔM15	Lutz & Bujard. 1997.
Plasmids		
pSET1	*S. suis* replicating plasmid with Cm resistance	Takamatsu *et al.*, 2005
pSET1 Δ*lac*	Deletion of a *lac* promoter in the pSET1	This study
pSETN1	pSET1 Δ*lac* carrying p15A replication origin	This study
p*ccpA*	pSET1 Δ*lac* p15A carrying *ccpA* and the putative native promoter	This study
pUHE212-1	IPTG-inducible expression vector with N-terminal six-His tag	Gamer *et al.*, 1992
pN-his *ccpA*	pUHE212-1 carrying *ccpA*	This study

[Tomoyasu *et al.*, 2010]

1 – 2 – 3. Generation of the *ccpA* knockout mutant and *cre* mutants.

A *ccpA* (DDBJ accession no. AB566422) knockout mutant (Δ*ccpA*) was produced by homologous recombination (Fig. 1A). Briefly, a 1,125-bp DNA fragment including the putative native promoter and coding region of *ccpA* (GenBank accession no. AB543256) was amplified by PCR from *S. intermedius* type strain NCDO2227 genomic DNA with primers ccpA EcoRI F and ccpA PstI R (Table 2). The 5' region of the *ccpA* DNA fragment (437 bp) was amplified by using ccpA EcoRI F and internal primer ccpA BamHI R (Table 2) and then digested with BamHI. The 3' region of the latter (525-bp) DNA fragment was amplified using internal primer ccpA SalI F and ccpA PstI R (Table 2) and then digested with SalI. The erythromycin resistance cassette (Em cassette) was amplified from *ily* knockout mutant UNS38 B3 [Sukeno *et al*., 2005] genomic DNA using primers erm (BamHI) F and erm (SalI) R (Table 2). The BamHI- and SalI-digested Em cassette was ligated to the BamHI-digested 5' region and SalI-digested 3' region of the 1,125-bp DNA fragment. This ligated fragment was amplified by nested PCR with the primers nested ccpA F and nested ccpA R (Table 2). UNS38 Δ*ccpA* was produced by transformation of competence-stimulating peptide (CSP; DSRIRMGFDFSKLF GK)-treated UNS38 with the PCR amplicon. Colonies were selected and isolated on a BHI agar plate containing 1 μg/ml Em. Disruption of *ccpA* was confirmed by PCR and immunoblotting using anti-CcpA mouse antiserum (Fig. 1B and Data not shown). For mutagenesis of the putative *cre* site located 3 bp behind the putative *ily* promoter −10 region (see Fig. 4A), an Em cassette was inserted 391 bp upstream from the *ily* translation start site (ATG). The procedure was performed as follows: the 1,408-bp DNA fragment within the region from bp 1799 to 391 upstream of the *ily* translation start site was amplified from UNS38 genomic DNA using primers ily up F and ily up R (Table 2). The amplified

fragment (*ily* UP) was digested with SalI. A 1,555-bp DNA fragment including the putative native promoter and a portion of the *ily* coding region was amplified from UNS38 genomic DNA using primers ily promo. F and ily No. 3 R (Table 2). The latter amplified fragment (*ily* no. 3) was digested with BglII. The BamHI- and SalIdigested Em cassette was ligated with SalI-digested *ily* UP and BglIIdigested 3' *ily* no. 3. The ligated fragment was then amplified by using primers nested PCR F and nested PCR R (Table 2). UNS38 Em resistance (EM^r) was produced by transformation of CSP-treated UNS38 with the PCR amplicon. Colonies were selected and isolated on a BHI agar plate containing 1 μg/ml Em. Insertion of the Em cassette was confirmed by PCR and sequencing (Data not shown). Mutations were introduced by PCR in the *cre* putative site (Fig. 4B). Primers mt-cre EcoRI F and mt-cre EcoRI R (Table 2) were designed to introduce mutagenesis into the putative *cre* site to create an EcoRI restriction site in the *cre* consensus sequence. A 3,918-bp DNA fragment of the *cre* upstream region including an Em cassette was amplified from UNS38 EM^r genomic DNA using primers ily up F and mt-cre EcoRI R (Table 2). A 1,119-bp DNA fragment of the *cre* downstream region was amplified from UNS38 EM^r genomic DNA using primers mt-cre EcoRI F and ily no. 3 R (Table 2). The amplified fragments were digested with EcoRI and ligated, and the resulting fragment was amplified using primers nested PCR F and nested PCR R (Table 2). UNS38 *creE* was produced by transformation of CSP-treated UNS38 with the PCR amplicon; UNS38 *creS* was produced similarly by using primers mt-cre SphI F and mt-cre SphI R (Table 2). The *cre* mutants were selected and isolated on a BHI agar plate containing 1 μg/ml Em. Introduction of mutations was confirmed by nucleotide sequencing (Data not shown).

Table 2: Oligonucleotides used in this study

Target or purpose	Primer or probe	Sequence (5'-3')
	PCR primers	
pSET1	pSET delta lac F	CTTGCATGCCTGCAGGTCGACTCTAGAG
	pSET delta lac R	CTCGCGCATGCGAAAGCCCCTAGAAGACG
p15A	p15A PstI	AGACTGCAGGGATATATTCCGCTTCCTCGC
	p15A SphI	ATCGCATGCGCTTGGACTCCTGTTGATAG
ccpA	ccpA EcoRI F	AAGAATTCAAAATGCTTGAAAGTGTTTCCAATAAGTG
	ccpA PstI R	GACTGCAGGCCTAACGGCCTCTTCTTTATTTCC
	his ccpA BamHI F	GAGGATCCATGAACACAGACGATACTG
	his ccpA PstI R	GCCCTGCAGTTATTTCCTTGTTGAACAACG
Disruption of *ccpA*	ccpA SalI F	CTATGGATGCGGTCGACTGTCTTGCAAAAGGCAACCG
	ccpA BamHI R	CTTTGGATCCAGCGTATTAACAACAGAGAC
	nested ccpA F	GTGATAAAATGATTTTGGTAGGAATGTGAAAACG
	nested ccpA R	GTTGAACAACGCTCAATCAGACCATGTGCC
Erythromycin cassette	erm (BamHI) F	AATGGATCCCCCGATAGCTTCCGCTATTG
	erm (SalI) R	CAGTAGTCGACCTAATAATTTATCTAC
Mutagenesis of *cre*	ily up F	AATACCAAGCCCAATGCAA CTCCTG
	ily up R	CTTTATAAGTCGACAGAAGCCCATTTTTCC
	ily promo. F	CTGTTTCTAACTAGATCTACTTCCCC
	ily No.3 R	GTAGTCTGTGTTGTTTTGGATAGTTGC
	nested PCR F	CGACACAATGACTAAAGTGTATCACTCATC
	nested PCR R	GAGATTGGTACAGCTGGACTTTGAGCAC
	mt-cre EcoRI F	GAAAGAATTCGCAATTTAGCAAAAGGAGGC
	mt-cre EcoRI R	GCGAATTCTTTCATTTATATATTAACACTATGATGAGC
	mt-cre SphI F	GAAAGCATGCAATTTAGCAAAAGGAGGC
	mt-cre SphI R	ATTGCATGCTTTCATTTATATATTAACACTATGATGAGC
qRT-PCR for *ily*	qRT-*ily* F	CTATTAGTGTAAACTTACCGGGATTG
	qRT-*ily* R	GGACTATTTGGAGAGTCTACGCTAGC
qRT-PCR for *gyrB*	qRT-*gyrB* F	GATGAGGCACTAGCAGGTTTTGC
	qRT-*gyrB* R	GTGAACAGTTGTCCCTGTTCG
Probes for EMSA	Probes	
	cre sense	ATAAATGAAAGCGTTAGCAATTTAGCAAA
	cre antisense	TTTGCTAAATTGCTAACGCTTTCATTTAT
	cre EcoRI sense	ATAAATGAAAGAATTCGCAATTTAGCAAA
	cre EcoRI antisense	TTTGCTAAATTGCGAATTCTTTCATTTAT
	cre SphI sense	ATAAATGAAAGCATGCAATTTAGCAAA
	cre SphI antisense	TTTGCTAAATTGCATGCTTTCATTTAT
	noncre sense	ATCGGTCTGTTATTTGTGTGTTTTTTATA
	noncre antisense	TATAAAAAACACACAAATAACAGACCGAT

[Tomoyasu *et al.*, 2010]

1 – 2 – 4. Complementation of the *S. intermedius* UNS38 Δ*ccpA* strain.

Streptococcus suis-E. coli shuttle vector pSET1 [Takamatsu *et al.*, 2001] was modified for complementation of the UNS38 Δ*ccpA* mutant. pSET1 was kindly supplied by T. Sekizaki (Research Center for Food Safety, The University of Tokyo). Primers pSET1 delta lac F and pSET1 delta lac R (Table 2) were designed to conduct the PCR for removal of the *lac* promoter and *lacZ*α for α-complementation in pSET1, creating SphI restriction sites in the resulting PCR product. The amplified fragment was digested with SphI, self-ligated, and transformed in *E. coli* C600. The resultant plasmid (pSET1 Δ*lac*) was digested with PstI and SphI. Primers p15A PstI F and p15A SphI R (Table 2) were designed to amplify a p15A replication origin of *E. coli* from pZA43 [Lutz & Bujard. 1997] by PCR creating PstI and SphI restriction sites in the resulting PCR product. This amplified fragment was digested with PstI and SphI, ligated to digested pSET1 Δ*lac*, and transformed into *E. coli* DH5αZ1 [Lutz & Bujard. 1997]. The resultant plasmid (pSETN1) was used for construction of the plasmid to complement UNS38 Δ*ccpA*. A *ccpA* fragment containing the putative native promoter was amplified by PCR using primers ccpA EcoRI F and ccpA PstI R (Table 2) from *S. intermedius* type strain NCDO2227 genomic DNA. The amplified fragment was digested with EcoRI and PstI and cloned into the corresponding sites in pSETN1. The resultant plasmid (p*ccpA*) was transformed into a CSP-treated UNS38 Δ*ccpA* mutant. Transformants were selected and isolated on a BHI agar plate containing 2 μg/ml Cm. Complementation of Δ*ccpA* was checked by PCR (Data not shown) and immunoblotting using anti-CcpA mouse antiserum (Fig. 1B).

1 – 2 – 5. Human erythrocyte agar plating.

Hemolysis induced by the bacterial cells was examined on human erythrocyte agar plates at 37°C

for 1 day under anaerobic conditions. Human blood was obtained from healthy Japanese volunteers and stored in sterilized Alsever solution at 4°C. Human blood cells (5 ml) in Alsever solution (5 ml) were washed three times with phosphate-buffered saline (PBS) by centrifugation (1,000 × *g*) and resuspended in 5 ml of PBS. PBS-suspended human erythrocytes were added to MOPS-BHI medium containing 1% agar at a final concentration of 10% (vol/vol).

1 – 2 – 6. Preparation of His-tagged recombinant CcpA.

The *ccpA* gene was amplified from the chromosomal DNA of *S. intermedius* type strain NCDO2227, using primers his ccpA BamHI F and his ccpA PstI R (Table 2). The amplified fragment was digested with BamHI and PstI and cloned into pUHE212-1. The resultant plasmid (pN-his *ccpA*) was transformed into *E. coli* DH5αZ1. Hyperexpression of the recombinant protein was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to *E. coli* cells in mid-log phase, and incubation continued at 37°C for 3 h. The cells were then harvested by centrifugation (5,000 × *g*) and resuspended in 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 20% sucrose, and 1 mg/ml lysozyme. The suspension was sonicated with an Astrason ultrasonic processor (model XL2020; Misonix Inc., Farmingdale, NY, USA) and diluted 5-fold with 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$. The resultant cell extract was centrifuged at 10,000 × *g* for 30 min to remove unbroken cells. The supernatant was loaded onto a Ni-nitrilotriacetic acid agarose column (Qiagen GmbH, Hilden, Germany) equilibrated with buffer A (20 mM Tris-HCl [pH 8.0], 300 mM NaCl, and 20 mM imidazole). Recombinant CcpA (N-his CcpA) was eluted with a linear gradient of 20 to 250 mM imidazole in buffer A. Peak fractions were diluted 10-fold with buffer B (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, and 10% glycerol) and loaded onto an Econo-Pac high-Q cartridge (Bio-Rad Laboratories, Richmond, CA). N-his

CcpA was eluted with a linear gradient of 0 to 1.0 M NaCl in buffer B, and the fractions were frozen at －80°C until use.

1 – 2 – 7. Anti-CcpA mouse antiserum and anti-ILY mouse monoclonal antibody.

To obtain anti-CcpA mouse antiserum, 50 μg purified N-his CcpA in 500 μl PBS was emulsified with an equal volume of Freund's complete adjuvant and administered into mice (intraperitoneal injection [i.p.]). Two weeks later, a booster shot of 50 μg of antigen was administered using Freund's incomplete adjuvant i.p. Mice were sacrificed at 1 week after the booster, and antisera were collected for immunoblotting. To obtain anti-ILY mouse monoclonal antibodies, 50 μg purified native ILY in 500 μl PBS was emulsified with an equal volume of Freund's complete adjuvant and administered into BALB/c mice i.p. Two weeks later, the same volume of the first booster shot of 50 μg of the antigen was administered using Freund's incomplete adjuvant i.p. After a further 2 weeks a final booster with 25 μg antigen in 100 μl PBS was administered to each mouse (intravenous injection [i.v.]). Three days after the final booster, mice were sacrificed and the spleen cells of each mouse were collected and used in hybridoma preparations using SP2/0-Ag14 myeloma as a hybridization partner according to the established PEG protocol [Nagamune *et al*., 2000]. Anti-ILY antibody-secreting hybridomas were screened by EIA in 1 μg ILY (fixed)/well in 96-well microtiter plates, and each hybridoma clone was established by the limiting dilution method as described previously [Nagamune *et al*., 2000]. Highly reactive monoclonal antibodies were selected and used in ILY immunoblotting

1 – 2 – 8. Electrophoretic mobility shift assay.

Biotin labeling of the 3' end of singlestrand oligonucleotides was performed by using a biotin 3' end DNA labeling kit according to the manufacturer's instructions (Thermo Fisher Scientific Inc.,

Rockford, IL). Biotin-labeled single-strand oligonucleotides (Table 2) were mixed and annealed with equal amounts of labeled complementary oligonucleotide (cre sense-cre antisense, cre EcoRI sense-cre EcoRI antisense, and cre SphI sense-cre SphI antisense). The mixture was incubated for 1 h at room temperature. Purified 100 nM N-his CcpA and a 5 nM biotin-labeled doublestranded oligonucleotide probe were mixed in a solution containing 2 μM nonlabeled double-stranded oligonucleotide (non-cre sense and non-cre antisense), 1 mg/ml bovine serum albumin, 7.5% glycerol, 5 mM $MgCl_2$, 1.5 mM EDTA, 1.5 mM dithiothreitol, 75 mM NaCl, 0.3% NP-40, and 15 mM Tris-HCl (pH 7.5) and then incubated at room temperature for 20 min. The DNA-protein complexes were separated from unbound probe on native 4% polyacrylamide gels which had been prerun in 0.25× Tris-borate-EDTA buffer (22.5 mM Tris-borate pH 8.3, 0.5 mM EDTA), followed by electroblotting to a positively charged nylon membrane. The blotted membrane was treated with a chemiluminescent nucleic acid detection module according to the manufacturer's instructions (Thermo Fisher Scientific Inc.) and exposed to a lumino-image analyzer LAS-4000 miniEPUV (Fuji Film, Tokyo, Japan).

1 – 2 – 9. Gel electrophoresis and immunoblotting.

S. intermedius cells were grown in BHI broth at 37°C under anaerobic conditions. The culture supernatant and cells were separated by centrifugation (5,000 × *g*). The cells were washed three times with PBS and resuspended in 1 ml or 0.5 ml of 20 mM HEPES-KOH, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol. Samples were then added to tubes containing lysing matrix B (Qbiogene Inc., Carlsbad, CA) and lysed in a FastPrep cell disruptor (Savant Instruments, Holbrook, NY). To obtain the soluble protein fraction, samples were centrifuged at 17,400 × *g* for 30 min and the supernatant retained. Total protein (10 μg) was subjected to sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the established Laemmli method [Laemmli. 1970]. For immunoblotting analysis, the gelresolved proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Blots were incubated with anti-CcpA mouse serum or anti-ILY monoclonal antibody and developed with an Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore) or 5-bromo-4-chloro-3'-indolylphosphate (BCIP)/nitroblue tetrazolium chloride (NBT) using horseradish peroxidase or alkaline phosphatase-conjugated anti-mouse immunoglobulin G as the secondary antibody.

1 – 3. Results

1 – 3 – 1. Effect of *ccpA* knockout on ILY secretion.

We introduced a *ccpA* knockout mutation into the UNS38 genome by insertion of an Em cassette in order to investigate whether CCR-controlled *ily* expression was observable in this mutant (Fig. 1A). To exclude the possibility that the mutant phenotypes result from other mutations in the chromosome, Δ*ccpA* was complemented in *trans* with a recombinant plasmid carrying *ccpA*, including its putative native promoter (p*ccpA*).

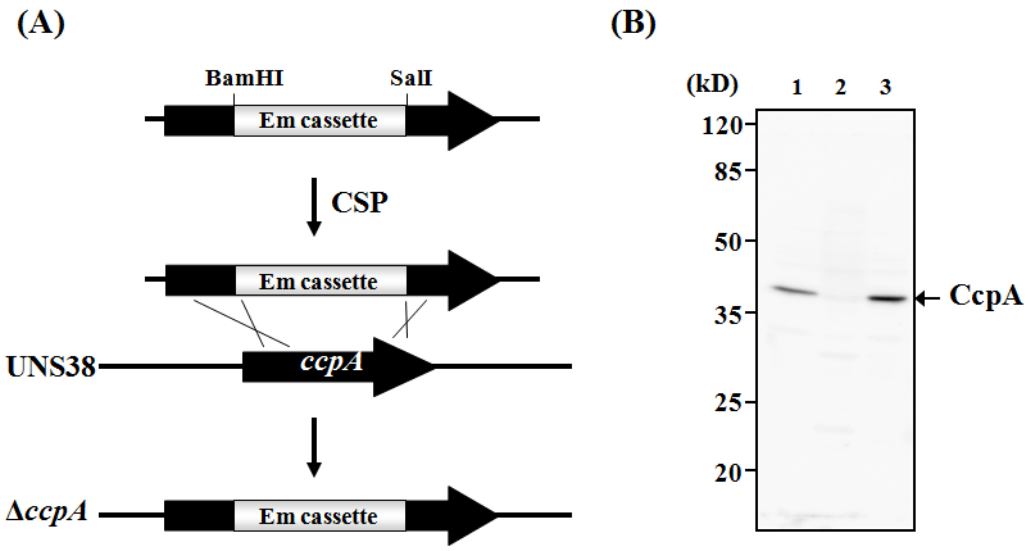

Fig. 1. (A) Schematic of the strategy for producing the Δ*ccpA* strain by allelic exchange mutagenesis; *ermF-ermAM*, erythromycin resistancegenes for the erythromycin cassette. (B) Immunoblotting analysis for confirmation of the Δ*ccpA* and the complementation of Δ*ccpA* mutant by *ccpA* (Δ*ccpA*+p*ccpA*). Whole-cell extracts (10 μg) were separated by 12.0% SDS-PAGE. Immunodetection was carried out with anti-CcpA mouse serum. Lane 1, UNS38; lane 2, Δ*ccpA*; lane 3, Δ*ccpA*+p*ccpA*.

[Tomoyasu *et al.*, 2010]

Immunoblotting analysis using anti-CcpA mouse antiserum was conducted to confirm the deletion of *ccpA* and complementation by p*ccpA* (Fig. 1B). Then UNS38, Δ*ccpA*, and the Δ*ccpA*

complementation strain (Δ*ccpA*+p*ccpA*) were inoculated onto human erythrocyte agar plates containing 0.1% or 1.0% glucose to confirm whether CcpA contributes to *ily* repression by CCR (Fig. 2A). When a human erythrocyte agar plate containing 0.1% glucose was used for the hemolysis assay, the sizes of the β−hemolytic zone surrounding the UNS38, Δ*ccpA*, and Δ*ccpA*+p*ccpA* cells were similar. However, in the presence of high levels of glucose (1.0%), ILY production was repressed in both UNS38 and Δ*ccpA*+p*ccpA* cells with a significantly reduced zone of β-hemolysis observed around the UNS38 cells. Conversely, a zone of hemolysis was observed around Δ*ccpA* cells even under high glucose culture conditions. We further examined the correlation between the amount of ILY secreted and the glucose concentration in the culture medium by using immunoblotting analysis (Fig. 2B). The amount of ILY secreted into the culture supernatant reduced with increasing glucose concentration in both UNS38 and Δ*ccpA*+p*ccpA* cells. On addition of more than 0.5% glucose to the culture medium, only a weak ILY signal was detected. Moreover, as shown in Fig. 2C, addition of the other preferred carbohydrates such as maltose and sucrose instead of glucose showed a similar repressive effect on ILY production. However, an apparent ILY signal was detected in the case of Δ*ccpA* even under high concentrations of glucose or other preferred and utilizable carbohydrates. Addition of a non-preferred carbohydrate such as glycerol did not repress ILY production in both UNS38 and Δ*ccpA* cells.

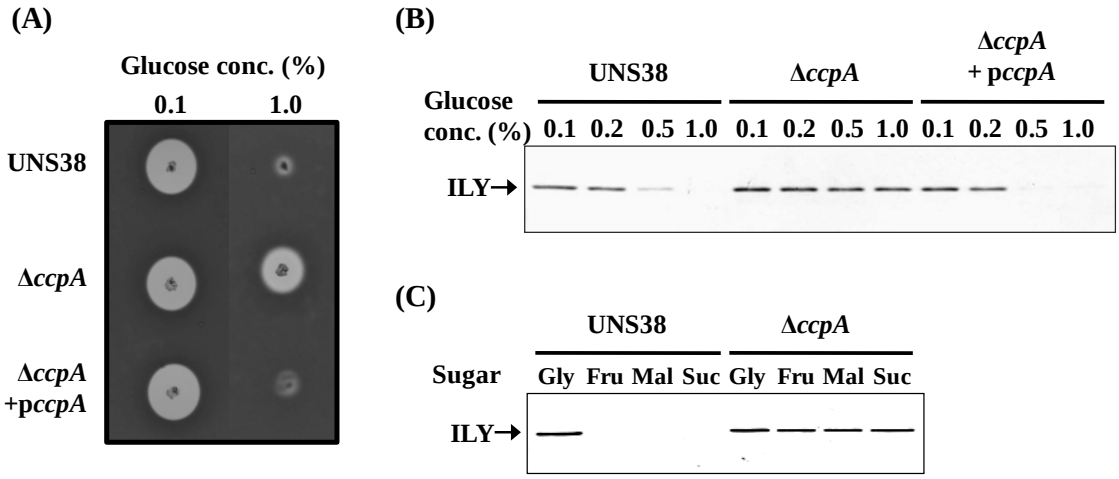

Fig. 2. Effects of glucose/utilizable carbohydrates on ILY secretion by Δ*ccpA* and Δ*ccpA*+p*ccpA* strains. (A) UNS38, Δ*ccpA*, and Δ*ccpA*+p*ccpA* cells were inoculated onto human erythrocyte agar plates containing 0.1 or 1.0% glucose and then incubated at 37° C for 1 day. (B) Cells were grown for 24 h at 37° C in MOPS–BHI medium containing different concentrations of glucose (0.1–1%). (C) UNS38, Δ*ccpA*, and Δ*ccpA*+p*ccpA* cells were grown for 18 h at 37° C in MOPS–BHI medium containing 0.1 % glucose and 1 % glycerol (Gly), fructose (Fru), maltose (Mal) or sucrose (Suc) and the optical density at 600 nm (OD_{600}) of the cultures was measured. Standardised amounts of the culture supernatants were analyzed by 12.0% SDS-PAGE. Anti-ILY monoclonal antibody was used as a probe for immunodetection of ILY.

[Tomoyasu *et al*., 2010]

During these experiments, we observed that Δ*ccpA* exhibited a slower growth rate compared to UNS38 and Δ*ccpA*+p*ccpA*. Therefore, the growth curve of these strains was examined by using BHI medium containing 0.1 or 1.0% glucose (Fig. 3A, B). UNS38 and Δ*ccpA*+p*ccpA* cells had an 8 h lag phase and then grew exponentially until 12 h in 0.1% glucose or 16 h in 1.0% glucose, following which they entered the stationary phase. However, the Δ*ccpA* cells had a prolonged lag phase (16 h in 0.1% glucose or 20 h in 1.0% glucose) and then grew exponentially (until 24 h in 0.1% glucose or 32 h in 1.0% glucose), following which they entered the stationary phase. Both the UNS38 and Δ*ccpA*+p*ccpA* cells grew with the same doubling time: around 70 min in 0.1% glucose and 60 min in 1.0% glucose. However, the doubling time of the Δ*ccpA* cells was relatively longer: 115 min in 0.1% glucose and 85 min in 1.0% glucose. These data suggested that CcpA might

control the cell growth rate to sense the extracellular glucose concentration.

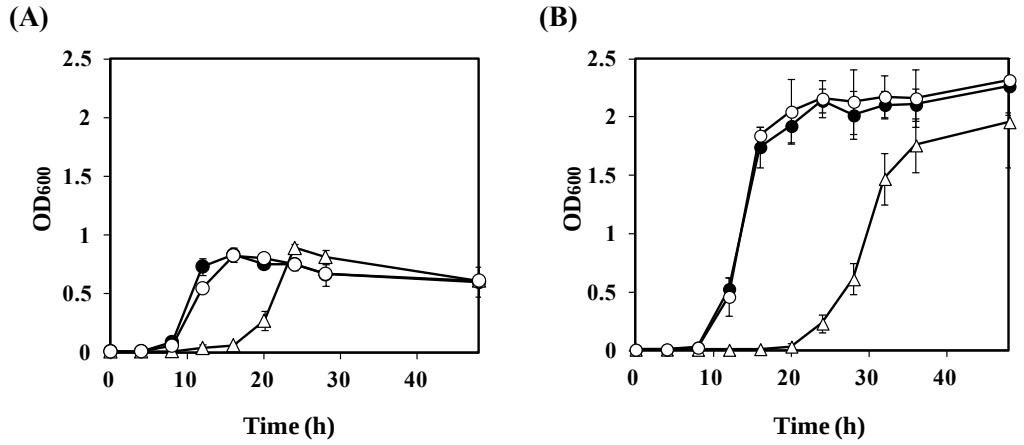

Fig. 3. Growth curves of UNS38, Δ*ccpA,* and Δ*ccpA*+p*ccpA* strains. Strains were cultured in BHI medium with 0.1% (A) or 1.0% (B) glucose, and the OD_{600} was measured at the indicated time points. Symbols: ●, UNS38; △, Δ*ccpA;* ○, Δ*ccpA*+p*ccpA.* The data represent the mean values ± standard deviation of at least 4 replicates each.

[Tomoyasu *et al.*, 2010]

1 – 3 – 2. Effect of *cre* mutation on ILY secretion.

Having demonstrated that *ily* expression was repressed by CcpA, we investigated to confirm that the putative *cre* site in the *ily* promoter region serves as a CcpA recognition element (Fig. 4A). Two *cre*-mutated strains were constructed to introduce point and deletion mutations into the *cre* site of UNS38 (Fig. 4B). UNS38 *creE* was mutated at three nucleotide positions and UNS38 *creS* had two nucleotide deletions and one point mutation in the *cre* site. These mutations did not cause the growth defect observed in the case of Δ*ccpA*. To estimate the level of ILY secretion, UNS38, UNS38 EM^r (Inserted Em cassette in front of the *ily* promoter region of UNS38 same as *cre* mutants), UNS38 *creE*, and UNS38 *creS* were inoculated onto human erythrocyte agar plates containing 0.1% or 1.0% glucose (Fig. 5A).

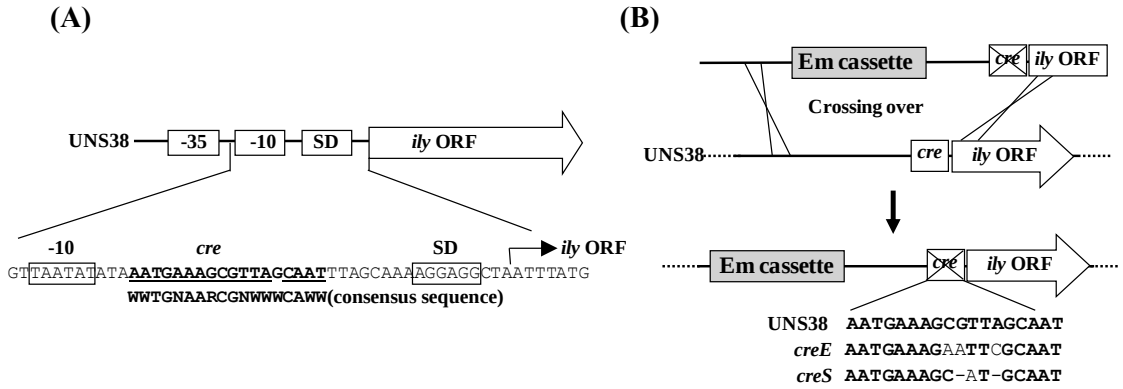

Fig. 4. (A) Schematic of a partial sequence of the UNS38 promoter region. The *cre*-like sequence of the *ily* promoter region and its consensus sequence are shown with bold letters, and the nucleotide sequences corresponding to the consensus sequence are underlined. *ily* ORF, *ily* open reading frame coding region; 10 and 35, *ily* promoter regions; SD, Shine-Dalgarno sequence. (B) Strategy for site-directed mutagenesis of *cre*. The EcoRI or SphI site was introduced by PCR in the *cre* consensus sequence using UNS38 EM^r (38 EM^r) genomic DNA (for details, see Materials and Methods). UNS38 *creE* (*creE*) and UNS38 *creS* (*creS*) were produced by transformation of CSP-treated UNS38, with each PCR amplicon carrying mutations in *cre*. In *creE*, the EcoRI site was introduced in the *cre* consensus sequence with three point mutations (plain text); in *creS*, the SphI site was introduced with two nucleotide deletions (-) and one point mutation (plain text).

[Tomoyasu *et al.*, 2010]

When human erythrocyte agar containing 0.1% glucose was used in hemolysis assay, the sizes of the surrounding β-hemolytic zones were similar in all cases. In contrast, high levels of glucose (1.0%) repressed ILY production in both UNS38 and UNS38 EM^r with only a small zone of β-hemolysis observed. However, a hemolytic zone was found around the colonies of the *cre* mutants even in the presence of 1.0% glucose. We further examined the correlation between the amount of ILY secreted and the concentrations of glucose or the other carbohydrate in the culture medium by immunoblotting analysis (Fig.5B and C).

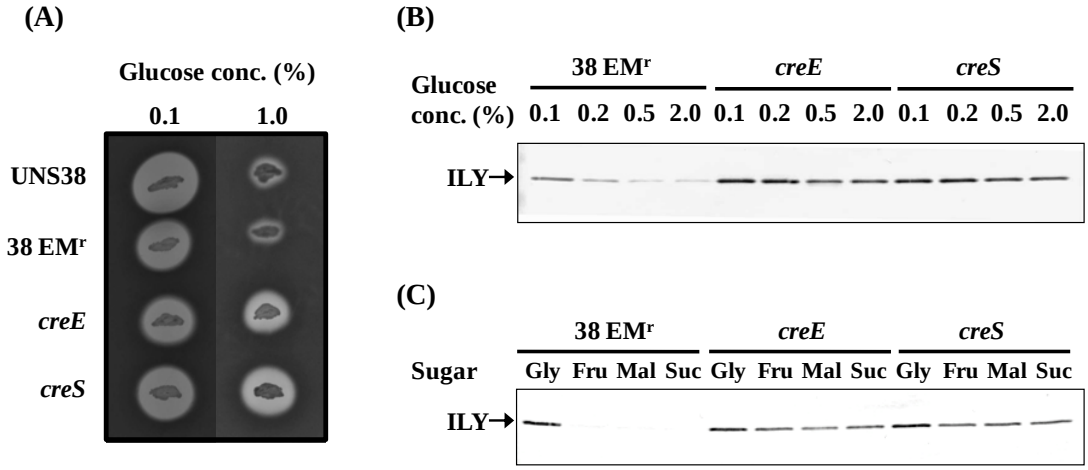

Fig. 5. Effects of glucose/utilizable carbohydrates on ILY secretion of the *cre* mutant strains. (A) UNS38, 38 EM^r, *creE*, and *creS* cells were inoculated onto human erythrocyte agar plates containing 0.1 or 1.0% glucose and then incubated at 37° C for 1 day. (B) Cells were grown for 20 h at 37° C in MOPS–BHI medium containing different concentrations of glucose (0.1–2%), (C) UNS38, 38 EM^r, *creE*, and *creS* cells were grown for 15 h at 37° C in MOPS–BHI medium containing 0.1 % glucose and 1 % glycerol (Gly), fructose (Fru), maltose (Mal) or sucrose (Suc). Then, the optical density at 600 nm (OD_{600}) of the cultures was measured and standardized amounts of the culture supernatants analyzed by 12.0% SDS-PAGE. Anti-ILY monoclonal antibody was used as a probe for immunodetection of ILY.

[Tomoyasu *et al.*, 2010]

The amount of ILY secreted into the culture supernatant reduced with increase of the concentration of glucose/utilizable carbohydrate in the UNS38 EM^r cells. Upon addition of more than 0.5% glucose/utilizable carbohydrate to the culture medium, only a weak ILY signal was detected. Conversely, an ILY signal was detected for both the *cre* mutants even under high concentrations of glucose/utilizable carbohydrate, as previously observed in Δ*ccpA* cells. These data strongly suggested that the *cre* site of the *ily* promoter region is responsible for the repression of *ily* expression through the association with CcpA.

1 – 3 – 3. Electrophoretic mobility-shift assay with *cre* or mutated *cre* DNA fragments.

To demonstrate that CcpA directly interacts with the *cre* region of the *ily* promoter, we performed electrophoretic mobility-shift assays with a purified, histidine-tagged *S. intermedius* CcpA protein (Fig. 6). To avoid non-specific binding of the biotin-labeled probes to CcpA, we carried out every

experiments in the presence of 2 μM non-specific double-stranded oligonucleotide; this amount was 400-fold higher than that present in the biotin-labeled probes. As a result, CcpA could induce a shift in the mobility of the DNA fragment containing the intact *cre* sequence. However, when fragments carrying a mutated *cre* sequence were used, a dramatic reduction was observed in the ability of CcpA to induce a shift. These data clearly showed that *ily* expression could be directly controlled by the binding of CcpA to the *cre* site of the *ily* promoter region.

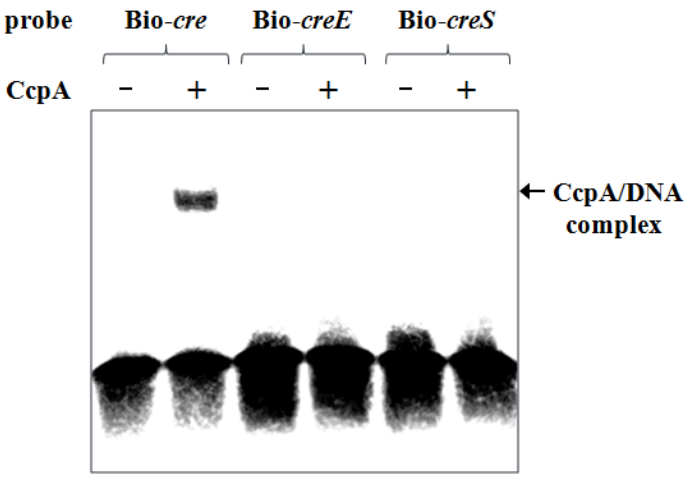

Fig. 6. Electrophoretic mobility shift assay results of biotin-labeled DNA fragments containing wild-type (Bio-*cre*) or mutated (Bio-*creE* or Bio-*creS*) *cre* sequences. Labeled DNA fragments (5 nM) were incubated with (+) or without (-) purified N-his CcpA (100 nM) and then electrophoresed on a 4% nondenaturing acrylamide gel. The labeled DNA was detected by chemiluminescence.

[Tomoyasu *et al.*, 2010]

2. LacR mutations are responsible for increased intermedilysin production and virulence

2 –1. Introduction

Oral bacteria can metabolize several sugars found in foods and drinks regularly consumed by humans. Lactose, a disaccharide formed from galactose and glucose, is most notably found in milk and other dairy products. This sugar plays an important role in oral microbial ecology and can contribute to the development of dental caries in both adults and young children. The metabolism of lactose and galactose in Gram-positive bacteria has been well characterized using Gram-positive cocci as models [Loughman & Caparon. 2007, van Rooijen RJ *et al*., 1991, Rosey *et al*., 1991, Zeng *et al.*, 2010]. It has been reported that these sugars are rapidly fermented by both the tagatose-6-phosphate (*lac*) and Leloir (*gal*) pathways in *Streptococcus mutans* strain UA159 [Zeng *et al.*, 2010]. The tagatose-6-phosphate pathway, known to be the most efficient route for lactose and galactose fermentation, is found almost exclusively in Gram-positive bacteria. Lactose and galactose fermentation can occur through these pathways. It has been reported that the enzymes involved in these pathways are encoded by the *lac* operon in some Gram-positive cocci [Loughman & Caparon. 2007, van Rooijen *et al*., 1991, Rosey *et al*., 1991, Zeng *et al.*, 2010]. LacR is a member of the GntR family of transcriptional regulators [van Rooijen *et al.*, 1992]. It has been shown that LacR can repress transcription of the *lac* operon by binding the LacR recognition element, which is direct repeats of the sequence TGTTTNWTTT (N = any base and W = A or T), on the *lac* promoter under lactose- or galactose-limited conditions [Barrière *et al.*, 2005, van Rooijen *et al.*, 1992]. It is believed that tagatose-6-phospate, a catabolite of galactose, can bind LacR and inhibit the interaction between LacR and the *lac* promoter. This allows RNA polymerase

to bind to the promoter and initiate transcription of the *lac* operon in lactose- or galactose-abundant conditions [van Rooijen *et al*., 1992, Zeng *et al.*, 2010].

Autoinducer 2 (AI-2) and CcpA reported to control the expression of *ily* [Pecharki *et al*., 2008, Tomoyasu *et al*., 2010]. However, the action of these two factors cannot explain the difference between strains with constitutively high production of ILY, which seem to be more highly pathogenic, and strains with low production of ILY. Therefore, we screened for additional factors that could regulate *ily* expression by employing random gene disruption in a low-ILY-producing PC574 strain from human dental plaque.

2 – 2. Material and Method

2 – 2 – 1. Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 3. *Streptococcus intermedius* was cultured at 37°C or 42°C under anaerobic conditions. Brain heart infusion (BHI) broth (Becton, Dickinson, Palo Alto, CA, USA) was used as the culture medium. Accumulation of lactate acidifies the culture medium and causes a loss of ILY activity in the culture supernatant [Tomoyasu *et al*., 2010]. Therefore, we used 3-(*N*-morpholino)propanesulfonic acid (MOPS)-buffered BHI (MOPS-BHI) medium for culture to monitor the amount of ILY secreted. The MOPS-BHI medium contained 100 mM MOPSbuffer (pH 7.4) and either 18.5 g/liter BHI broth or 17.5 g/liter BHI broth without dextrose (United States Biological, Swampscott, MA, USA). MOPS-BHI medium was supplemented with glucose or other sugars at specified concentrations. *E. coli* cells were grown in Luria- Bertani (LB) medium at 37°C under aerobic conditions. The following antibiotics were added at the following concentrations: ampicillin, 100 μg/ml for *E. coli*; chloramphenicol (Cm), 20 μg/ml for *E. coli* and 2 μg/ml for *S. intermedius*; and erythromycin (Em), 100 μg/ml for *E. coli* and 1 μg/ml for *S. intermedius*.

2 – 2 – 2. Random gene disruption of low-ILY-producing strain PC574.

pGh9:IS*S1* (Table 3) was transformed into *S. intermedius* PC574 cells that had been treated with competence-stimulating peptide (CSP) (DSRIRM GFDFSKLFGK), which were then cultured on BHI agar with Em for plasmid selection at 42°C. Around 5,000 colonies were transferred with toothpicks to human erythrocyte agar. Three independent high-ILY-producing strains (PC574 IS*S1* 1 to 3), which could generate larger beta-hemolysis zones than strain PC574 on human erythrocyte agar, were used for plasmid rescue experiments.

Table 3: Bacterial strains and plasmids used in this study

Strains	Relevant characteristics	Reference or source
S. intermedius		
PC574	ILY low-producing strain from human dental plaque	Nagamune *et al.* 2000
UNS38	ILY high-producing strain from human brain abscess	Sukeno *et al.* 2005
UNS38 B3	*ily* knockout strain derived from UNS38	Sukeno *et al.* 2005
PC574 Δ*lacR*	*lacR* knockout strain derived from strain PC574	This study
E. coli		
DH5αZ1	F$^-$ Φ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1 tetR lacI*^{q} Spec^r	Lutz & Bujard. 1997
TG1(der)	F' *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$*/recA*::*tet supE thi* Δ(*lac-proAB*) *km-repA*	Lun & Willson. 1992
Plasmids		
pGh9:IS*S1*	Generate random insertions into the chromosome	Maguin *et al.* 1996
pSETN1	*Streptococcus-E. coli* shuttle vector	Tomoyasu *et al.* 2010
p*lacR*	pSETN1 carrying *lacR* isolated from NCDO2227	This study
p*lacR*(C135Y)	pSETN1 carrying *lacR* containing a cysteine 135-to-tyrosine mutation isolated from P22	This study
p*lacR*(S117N, C135Y)	pSETN1 carrying *lacR* containing a serine 117-to-asparagine and cysteine135-to-tyrosine mutation isolated from HW7	This study
p*lacR*(R37L)	pSETN1 carrying *lacR* containing an arginine 37-to-leucine mutation isolated from A4676a	This study
p*lacR*(L48F)	pSETN1 carrying *lacR* containing a leucine 48-to-phenylalanine mutation isolated from UNS46	This study
p*lacR*(V21D)	pSETN1 carrying *lacR* containing a valine 48-to-aspartic acid mutation isolated from UNS38	This study
p*lacR*(R50W)	pSETN1 carrying *lacR* containing an arginine 50-to-tryptophan mutation isolated from UNS35	This study
p*lacR*(S117I)	pSETN1 carrying *lacR* containing a serine 117-to-isoleucine isolated from UNS32	This study
p*lacR*(V30A)	pSETN1 carrying *lacR* containing a valine 30-to-alanine mutation isolated from UNS45	This study
p*lacR*(42Q_44Ldup, C135Y)	pSETN1 carrying *lacR* containing duplication of a glutamine 42-to-leucine 44 and cysteine135-to-tyrosine mutation isolated from JICC 33616	This study

[Tomoyasu *et al.*, 2013]

2 – 2 – 3. Plasmid rescue method.

Sequences flanking the pGh9:IS*S1* insertion site were obtained using a sequence rescue strategy, as described previously [Lun & Willson. 2005]. Briefly, the chromosomal DNA of *S. intermedius* PC574 IS*S1* 1 was purified and digested with EcoRI. The digested DNAwas self-ligated and then introduced into *E. coli* TG1 (der) cells. The recombinant plasmids were purified, and the chromosomal DNA regions corresponding to these plasmids were amplified by PCR and sequenced using the primerspGh^{+}9#02 and 5'IS*S1*(rev) (rev stands for reverse) [Lun & Willson. 2005]. Alignment of the PCR product sequences bridging the transposition site and the *S. intermedius* NCDO2227 genome sequence (GenBank accession no. AP010969) helped identify the chromosomal sequence flanking the transposition site.

2 – 2 – 4. Databases and sequence alignment.

Nucleotide and protein sequences were obtained from the Microbes genomic BLAST databases by an Entrez cross-database search at the National Center for Biotechnology Information (NCBI) (National Institutes of Health, USA). The degree of homology between the *lac* operon from *S. intermedius* NCDO2227 and the consensus sequences of the LacR recognition element was determined using the software program GENETYX-MAC version 17. Sequence alignments between LacR sequences from the type strain NCDO2227 and the strains isolated from clinical specimens or dental plaques were performed using the NCBI BLAST Needleman-Wunsch Global Sequence Alignment Tool.

Table 4: Oligonucleotides used in this study

Purpose	Name	Sequence (5'-3')
Disruption of *lacR*	lacR F	GAGGCGTTGAACTGATACATTTTCGAC
	lacR BamHI R	TGCGGATCCAGTTCTTGAAGAATAACTC
	lacR SalI F	AATGTCGACCGTATACGCGTGTGTTATAG
	lacR R	GATTTTCATCGTACTCATTACCCAATC
	erm BamHI F	AATGGATCCCCCGATAGCTTCCGCTATTG
	erm SalI R	CAGTAGTCGACCTAATAATTTATCTAC
Complementation of Δ*lacR* mutant and 6his-tagged *lacR*	lacR EcoRI F	CAAGAATTCGGCGTAAAGCTCCACGTTGG
	lacR BamHI F	GAGGATCCATGAAAGAAGGACGACATAGAG
	lacR PstI R	AAAATCACCTGCAGCTTCACGAACAGGTG
Nucleotide sequences of *lacR*	lacR seq. F	CTTGTTTTGTTGTCATTCCCAGACTCC
	lacR seq. R	CAGGCTCAATCTAACATAGATGAGACCTG
	lacR seq. F1	GGAATCTAATTATATGATTAGAAAGGAG
	lacR seq. R2	GTCAATCTTTCTTCAAAAAAATCACCTGC
Biotinylated DNA probe pull-down assay	Bio-Pily F	Bio-TAGCCGCTTTATCCATCTAACTCTTATCCC
	Pily R	AAATTAGCCTCCTTTTGCTAAATTGCTAAC
	Bio-PlacD F	Bio-TTTGTCTCCTTTCTAATCATATAATTAG
	PlacD R	TCCCAGACTCCTTTTATTTTATATGATTTC
	Bio-PlacA F	Bio-ATCCTCTCCTTCTGTTTATTTGTGTTG
	PlacA R	TGTTATACCTCCTTTTTCTTTTACAACAAC
	Bio-lacF F	Bio-GAATAGGGAAGAAACAACATTACTTGG
	lacF R	CAGTAAATCAGTCTGTGCACGATGCGCGTC

[Tomoyasu *et al.*, 2013]

2 – 2 – 5. Generation of *lacR* knockout mutant in strain PC574.

A *lacR* knockout mutant (Δ*lacR* mutant) was produced by homologous recombination as shown in Fig. 7A. Briefly, the 5' region of the *lacR* DNA fragment (533 bp) was amplified using primer lacR F (F stands for forward) and internal primer lacR BamHI R (R stands for reverse) (Table 4) and then digested with BamHI. The 3' region of the latter (560-bp) DNA fragment was amplified using the internal primers lacR SalI F and lacR R (Table 4) and then digested with SalI. The Em resistance cassette was amplified from the genomic DNA of *ily* knockout mutant UNS38 B3 [Sukeno *et al*., 2005] using primers erm BamHI F and erm SalI R (Table 4). The BamHI- and SalI-digested Em cassette was ligated to the BamHI-digested 5' region and SalI-digested 3' region, and the ligated fragment was then amplified by PCR with primers lacR F and lacR R (Table 4). The amplified fragment was used to construct the Δ*lacR* mutant. The Δ*lacR* mutant was produced by transformation of CSP-treated *S. intermedius* PC574 cells with the PCR amplicon. Colonies were selected on BHI agar containing 1 μg/ml Em. Disruption of *lacR* was confirmed by PCR, as well as by immunoblotting using anti-LacR rabbit antiserum (Fig. 7B).

2 – 2 – 6. Complementation of *S. intermedius* PC574 Δ*lacR* mutant.

Streptococcus-*E. coli* shuttle vector pSETN1 [Tomoyasu *et al.*, 2010] was used for complementation of the *S. intermedius* PC574 Δ*lacR* mutant. *lacR* fragments containing the putative native promoter were amplified by PCR using the primers lacR EcoRI F and lacR PstI R (Table 4) from *S. intermedius* type strain NCDO2227 and genomic DNA from the clinically isolated strains A4676a, UNS46, UNS38, UNS35, UNS32, UNS45, JICC 33616, HW7, and P22. The amplified fragments were digested with EcoRI and PstI, cloned into the corresponding sites in pSETN1, and transformed into *E. coli* DH5αZ1. Each resultant plasmid (Table 3) was transformed

into a CSP-treated PC574 Δ*lacR* mutant. Transformants were selected and isolated on BHI agar containing 2 μg/ml Cm and then confirmed by immunoblotting using anti-LacR rabbit antiserum, PCR, and reverse transcription (RT)-PCR (Data not shown). Hemolysis assays were used to monitor the ability of these plasmids to complement the Δ*lacR* mutant.

2 – 2 – 7. qRT-PCR analysis.

S. intermedius cells were grown in the MOPS-BHI medium at 37°C for 16 h under anaerobic conditions, and the cells were subsequently separated by centrifugation (5,000 × *g*). Isolation of total RNA from cells and quantitative RT-PCR (qRT-PCR) analysis was performed as previously described [Tomoyasu *et al*., 2010]. Real-time PCR was performed in 96-well plates using an ABI PRISM 7900HT instrument with *Power* SYBR green master mix (Applied Biosystems, Warrington, United Kingdom). The primer set of qRT-*ily* F and qRT-*ily*R [Tomoyasu *et al.*, 2010] was used for quantification of *ily* mRNA. The primer set of qRT-*gyrB* F and qRT-*gyrB* R [Tomoyasu *et al*., 2010] was used as an internal control to normalize the amount of total RNA in each sample. To plot calibration curves for the primer set, cDNA from the *S. intermedius* PC574 Δ*lacR* mutant was used as the template in a 5-step dilution process (corresponding to 100, 50, 25, 12.5, and 6.25 ng of input RNA). Thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles, with 1 cycle consisting of 15 s at 95°C and 1 min at 60°C. The amounts of target RNAs were calculated from the calibration curves.

2 – 2 – 8. Infection assay.

S. intermedius cells were grown in BHI broth at 37°C for 20 h under anaerobic conditions. The infection assays were performed as previously described with minor modifications [Sukeno *et al*., 2005, Tomoyasu *et al*., 2012]. HepG2 cells in 350 μl of Dulbecco's modified Eagle's medium

(DMEM) containing 10% fetal bovine serum (FBS) without antibiotics were dispensed into 48-multiwell tissue culture plates (1×10^5 cells/well) and cultured overnight at 37°C in the presence of 5% CO_2. For cell infection, bacterial cultures were centrifuged at 13,000 × *g* for 1 min, and the cells were resuspended at a density of 1×10^6 cells in 350 μl of DMEM in the absence of antibiotics containing 5% FBS and 0.1% heat-inactivated human plasma from a healthy Japanese volunteer. The bacterial suspension was added to the HepG2 cells, and infection was allowed to proceed for 3 h in the 48-multiwell tissue culture plates. The supernatant was then completely removed, and cells were washed three times with PBS. Infected cells were cultured in 350 μl of fresh medium containing 5% FBS and 0.1% human plasma. A portion of the culture medium (200 μl) was replaced with fresh medium every 12 h to avoid accumulation of ILY. The viability of infected cells was determined using the neutral red (NR) method [Borenfreund & Puerner. 1985]. After infection, the medium was removed at the indicated time point, and the cells were incubated with 350 μl of NR solution (50 μg/ml) in DMEM for 3 h at 37°C. The cells were subsequently washed three times with PBS and then fixed with 200 μl formaldehyde solution (1.0%, vol/vol) containing 1 mM HEPES-KOH (pH 7.3), 0.85% NaCl, and 1.0% $CaCl_2$. To extract the dye taken into viable cells, the fixed cells were lysed with 1% acetic acid in 50% (vol/vol) ethanol. The absorbance was then measured at 540 nm (A_{540}). The control for 0% viability consisted of cells exposed to 1.0 M HCl, while the control for 100% viability consisted of cells incubated in bacterium-free DMEM. The level of cytotoxicity was calculated as follows: viability (expressed as a percentage) = [(A_{540} of the extract from infected cells − A_{540} of the extract from the control for 0% viability)/(A_{540} of the extract from the control with 100% viability − A_{540} of the extract from the control for 0% viability)] × 100.

2 – 2 – 9. Human erythrocyte agar plating.

Hemolysis induced by the bacterial cells was examined on human erythrocyte agar according to the method described previously [Tomoyasu *et al*. 2010].

2 – 2 – 10. Hemolysis assay.

Hemolysis was carried out using previously described method [Nagamune *et al.*, 1996, Tomoyasu *et al*. 2010]. *S. intermedius* cells were grown in MOPS-BHI medium containing 1% (wt/vol) glucose, galactose, or lactose at 37°C for 48 h under anaerobic conditions. The culture supernatant was obtained by centrifugation (5,000 × *g*) and standardized by dilution with PBS for an optical density at 600 nm (OD_{600}) of 0.25 to 0.5 for the assay. Human erythrocytes stored in sterilized Alsever's solution were washed three times with PBS at 4°C by centrifugation (1,000 × *g*) before use. Chilled PBS containing 5×10^7 erythrocytes/ml and the dilution series (12.5- to 1,600-fold) of the culture supernatant with PBS were mixed in microcentrifuge tubes (total volume of 0.5 ml). Incubation was at 37°C for 1 h. After the reaction, nonlysed erythrocytes were removed by centrifugation (1,000 × *g*) at 4°C for 5 min. The A_{540} of 200 µl of the supernatant was measured in a microplate reader (model 550; Bio-Rad, Hercules, CA, USA). The percent hemolysis was calculated as follows: percent hemolysis = [(A_{540} of the supernatant from the sample containing diluted culture supernatant − A_{540} of the supernatant from the sample containing no diluted culture supernatant)/(A_{540} of the supernatant from the sample completely hemolyzed by hypotonic processing − A_{540} of the supernatant from the sample containing no diluted culture supernatant)] × 100. The relative hemolytic activity was calculated as follows: relative hemolytic activity (as a percentage) = (dilution rate of culture supernatant sample giving 50% of hemolysis/dilution rate of culture supernatant of *S. intermedius* UNS38 or PC574 Δ*lacR* mutant giving 50% of hemolysis)

× 100.

2 – 2 – 11. Preparation of His-tagged recombinant LacR.

lacR was amplified from the chromosomal DNA of *S. intermedius* type strain NCDO2227 by using the primers lacR BamHI F and lacR PstI R (Table 4). The amplified fragment was digested with BamHI and PstI and cloned into pUHE212-1. The resultant plasmid (pN-his *lacR*) was transformed into *E. coli* DH5 Z1. Hyperexpression of the recombinant protein was induced by adding 1 mM IPTG to *E. coli* cells. The cells were then harvested by centrifugation (5,000 × *g*) and resuspended in buffer A (20 mM Tris-HCl buffer pH 8.0 containing 300 mM NaCl, 20 mM imidazole, and 6 M urea). The suspension was sonicated using an Astrason ultrasonic processor and then incubated at 30°C for 1 h to denature the proteins. The resultant cell extract was centrifuged at 10,000 × *g* for 20 min to remove unbroken cells. The supernatant was loaded onto a nickelnitrilotriacetic acid (Ni-NTA) agarose column (Qiagen, Valencia, CA, USA), and the column was washed with buffer A. Proteins bound to the column were eluted with a linear gradient of 20 to 500mMimidazole in 20 mM Tris-HCl pH 8.0 containing 300 mM NaCl and 6 M urea. Peak fractions were dialyzed with 20 mM Tris-HCl buffer pH 8.0 containing 100 mM NaCl, 1 mM EDTA, and 10% glycerol.

2 – 2 – 12. Anti-LacR rabbit antiserum.

To obtain anti-LacR rabbit antiserum, 150 µg of purified His-tagged recombinant LacR in 1.5 ml of PBS was emulsified with an equal volume of Freund's complete adjuvant and administered to rabbits (intramuscular injection). Three booster shots of 150 µg of the antigen were administered using Freund's incomplete adjuvant (subcutaneous injection) at 3-week intervals. Ten milliliters of blood was drawn 2 weeks after the final booster shot was administered, and antiserum was collected for immunoblotting.

2 – 3. Results

2 – 3 – 1. Identification of a factor that represses *ily* expression.

We screened for another factor regulating *ily* expression by performing random gene disruption of a low-ILY-producing strain (PC574) from human dental plaque using plasmid pGh9:IS*S1* with a thermosensitive replicon and transposable element [Maguin *et al.*, 1996]. By culturing plasmid-transformed cells at 42°C, the plasmid integrated into the chromosome and disrupted the gene at random locations. Three independent high-ILY-producing colonies were identified after we observed the degree of hemolysis produced by approximately 5,000 colonies on human erythrocyte agar. Using a plasmid rescue method, the position of integration of the plasmid in one high-ILY-producing strain was determined to be at nucleotide 588 of a 747-bp open reading frame sharing the highest homology with *lacR* of *Streptococcus anginosus*.

2 – 3 – 2. Construction and characterization of a Δ*lacR* mutant and its complementation strain.

In order to confirm that a *lacR* mutation is responsible for the high-ILY-producing phenotype, a *lacR* knockout mutation was introduced into the *S. intermedius* PC574 genome through insertion of an Em cassette. To exclude the possibility that the mutant phenotypes resulted from other mutations in the chromosome, the Δ*lacR* mutant was complemented in *trans* with a recombinant plasmid carrying *lacR* and its putative native promoter (p*lacR*). Immunoblotting analysis using anti-LacR rabbit antiserum was conducted to confirm the deletion of *lacR* and complementation by p*lacR* (Fig. 7B).

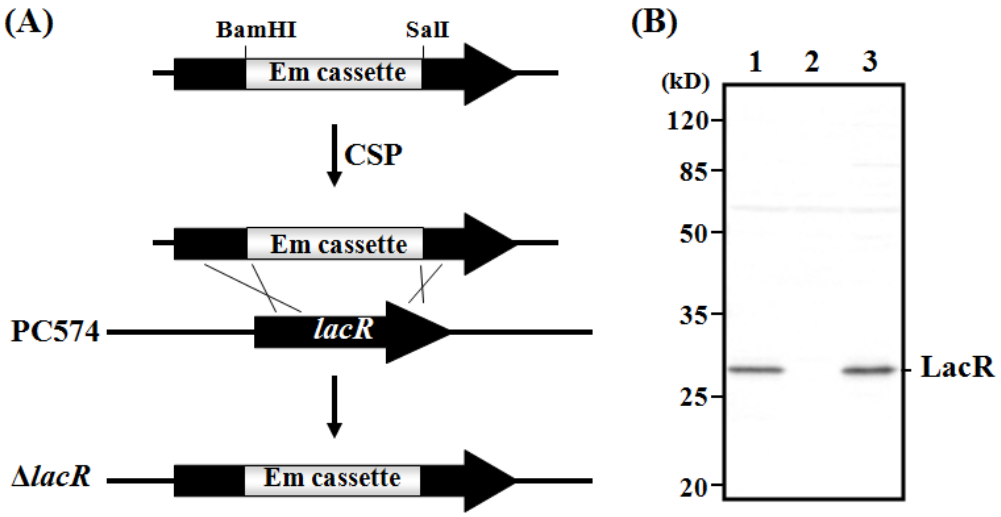

Fig. 7. (A) Schematic illustration of the strategy for producing the Δ*lacR* mutant by allelic exchange mutagenesis. (B) Immunoblotting analysis for confirmation of the disruption of *lacR* and its plasmid complementation. PC574 containing control vector pSETN1 (PC574+pSETN1), PC574 Δ*lacR* containing control vector (Δ*lacR*+pSETN1), and PC574 *lacR* mutant transformed with p*lacR* (Δ*lacR*+p*lacR*). Whole-cell extracts (10 μg) were separated by SDS-PAGE. Immunodetection was carried out with anti-LacR rabbit serum. Lane 1, PC574+pSETN1; lane 2, Δ*lacR*+pSETN1; lane 3, Δ*lacR*+p*lacR*.

[Tomoyasu *et al.*, 2013]

The hemolytic activities of these strains were examined on human erythrocyte agar (Fig. 8A). The Δ*lacR* mutant formed a larger β-hemolytic zone than the wild-type cells. Only a small zone of β-hemolysis, similar in extent to that of the wild-type cells, was observed around the *lacR* complementation cells. Therefore, we further examined the amount of ILY secreted in the culture supernatant by hemolysis assays for *S. intermedius* PC574, Δ*lacR* mutant, and the complemented strain (Fig. 8B).

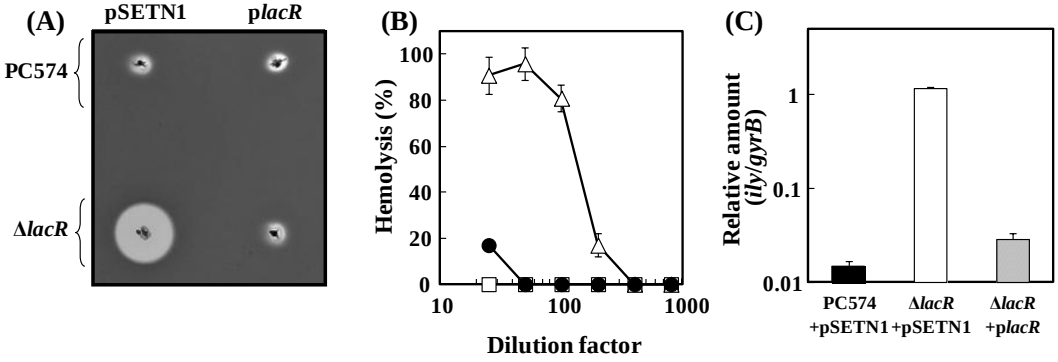

Fig. 8. Hemolysis and *ily* transcriptional activity of Δ*lacR* strain
Hemolytic activity on human erythrocyte agar (A). PC574 and PC574 Δ*lacR* strain transformed with pSETN1 or p*lacR* (WT) were inoculated onto human erythrocyte agar, and then incubated at 37 for 1 day. Hemolytic activity of the culture supernatant (B). Cells were grown for 48 h at 37° C in MOPS-BHI medium containing 0.1% glucose. Culture supernatant standardized at $OD_{600\ nm}$ was diluted from 25- to 800-fold by 2-fold serial dilutions, and the cytolytic activity of ILY in the diluted culture supernatant was estimated by hemolysis assay. The results are plotted on a logarithmic scale in the horizontal axis. Symbols: ●, PC574 pSETN1; △, PC574 Δ*lacR* pSETN1; □, PC574 Δ*lacR* p*lacR*. Relative expression levels of *ily* (C). The *ily* expression levels in PC574+pSETN1, Δ*lacR*+pSETN1, and Δ*lacR*+p*lacR* are indicated relative to the *gyrB* expression level. The results are plotted on a logarithmic scale in the vertical axis. The data represent the mean values ± standard deviation of 6 replicates each.

[Tomoyasu *et al.*, 2013]

As expected, Δ*lacR* mutant cells secreted higher levels of ILY than the wild-type cells, and *lacR* complementation reduced ILY secretion to the level of the wild-type cells. The higher level of ILY secretion by the Δ*lacR* mutant into the culture medium was also confirmed by immunoblotting using anti-ILY antibody (Data not shown). We also compared the level of *ily* mRNA in *S. intermedius* PC574, Δ*lacR* mutant, and the complemented strain by qRT-PCR, and measured the relative amounts of *ily* mRNA (level of expression of *ily* compared to the level of expression of *gyrB*) in these strains (Fig.8C). The expression level of *ily* in Δ*lacR* mutant cells was 80.7-fold greater than that in PC574 cells and was reduced to a level similar to that in PC574 by p*lacR* complementation.

2 – 3 – 3. Effects of sugars on *ily* expression.

The ability of LacR to repress transcription of the *lac* operon is believed to derive from its ability to interact with the *lac* promoter. This interaction, and hence function, can be blocked by LacR binding to tagatose-6-phospate which is a catabolite of lactose and galactose. Because our data strongly suggest that LacR represses *ily* expression, derepression may occur in the presence of lactose or galactose in the culture medium. To confirm this possibility, PC574 cells were cultured in MOPS-BHI medium supplied with 0.1% glucose, lactose, or galactose. The amount of ILY secreted into the culture supernatant increased with the addition of lactose or galactose as a carbon source for PC574 cells (Fig. 9).

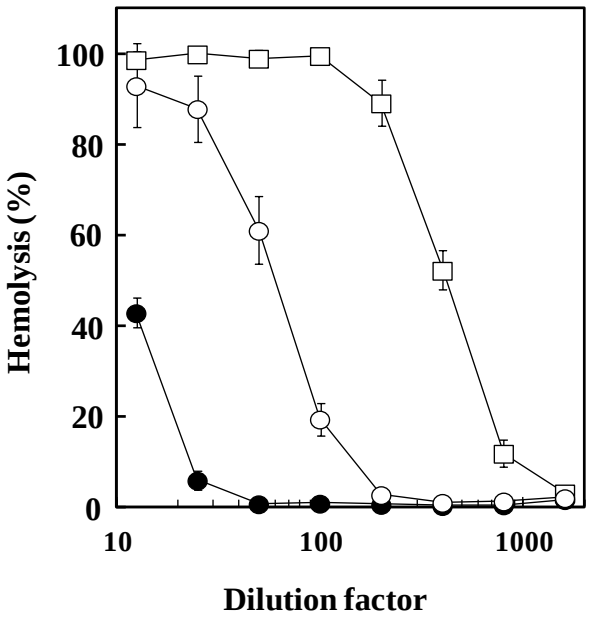

Fig. 9. Effects of sugars on ILY secretion PC574 was grown for 48 h at 37° C in MOPS-BHI medium containing 0.1% glucose (●), lactose (○), or galactose (□). Culture supernatant standardized at OD_{600} was diluted from 12.5- to 1,600-fold by serial 2-fold dilutions, and then the hemolytic activity was measured. The data in the graph are the mean ± SD of 4 replicates of independent experiments.

[Tomoyasu *et al.*, 2013]

The cells cultured in galactose-supplemented medium secreted larger amounts of ILY than those

cultured in the medium with lactose. It is possible that glucose produced by hydrolysis of lactose can repress *ily* expression by catabolite control repression with CcpA, and this might account for the difference in ILY secretion between the lactose- and galactose-supplemented cases. We also confirmed these results by immunoblotting using anti-ILY antibody (Data not shown). These data clearly showed that *ily* expression was regulated by LacR monitoring of extracellular galactose -containing sugars in the growth environment.

2 – 3 – 4. Cytotoxicity of Δ*lacR* mutant on human liver HepG2 cells.

ILY is considered to be a major virulence factor of *S. intermedius*, which is essential for invasion of and cytotoxicity to human cells [Sukeno *et al*., 2005]. It was observed that Δ*lacR* cells secreted higher amounts of ILY when compared with the wild-type cells, suggesting that this mutation may result in increased cytotoxicity to human cells. Therefore, we examined the cytotoxicity of Δ*lacR* and the complemented strain on the human hepatoma cell line HepG2 (Fig. 10). With Δ*lacR*, viability of the HepG2 cells was markedly reduced after infection, and almost all HepG2 cells were killed after 2 days. However, by comparison, PC574 cells and the complemented strain showed only slight cytotoxicity toward HepG2 cells with approximately 60% of the HepG2 cells surviving 3 days after infection. These data clearly show that disruption of *lacR* in *S. intermedius* causes an increase in cytotoxicity, compared to the parental strain, through increased ILY production.

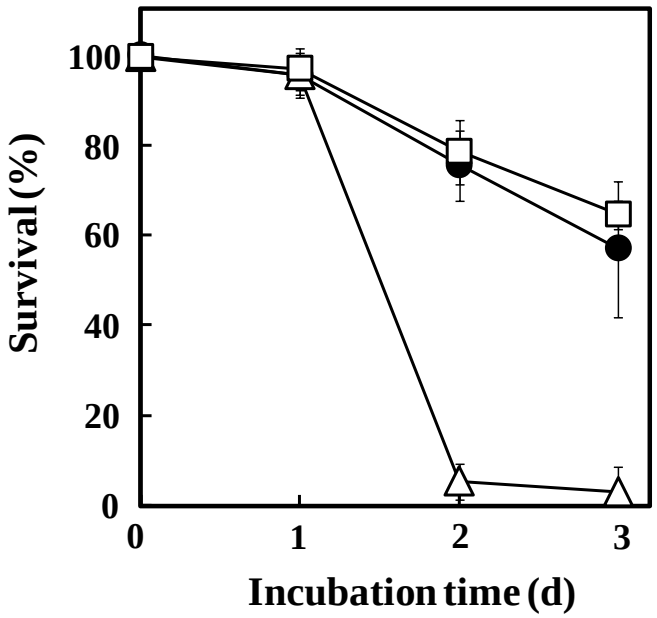

Fig. 10. Cytotoxic effect of Δ*lacR* and its complemented strain on HepG2 cells
Cytotoxic effects were observed over 3 days post bacterial infection.
Symbols: ●, PC574+pSETN1; △, Δ*lacR*+pSETN1; □, PC574 Δ*lacR*+p*lacR*.
The data in the graph are the mean ± SD of 5 replicates of independent experiments.

[Tomoyasu *et al.*, 2013]

2 – 3 – 5. Correlation between ILY production and mutation of LacR in clinical isolates.

The results shown thus far strongly suggest that hyper-production and secretion of ILY in Δ*lacR* should lead to increased pathogenicity in *S. intermedius*. Therefore, we investigated the hemolytic activity and nucleotide sequences of *lacR* from 50 strains isolated from clinical specimens, 7 strains from dental plaques and the type strain NCDO2227, to determine the possible correlations between ILY production and mutations in LacR. We classified 13 strains from the 50 strains isolated from clinical specimens as ILY high-producing strains and determined that these could produce >30% ILY compared to ILY high-producing strain UNS38 (Table 5). Almost all of the ILY high-producing strains were from serious, deep-seated abscesses.

Table 5: Mutation in LacR and relative hemolytic activity of clinically isolated ILY high-producing strains

Strains	Isolation source	Mutation in LacR	Relative hemolytic activity [a] (%)
A4676a	Brain abscess	R37L	329.9 ± 13.3
UNS46	Liver abscess	L48F	187.0 ± 18.2
JICC 33405	Empyema, mediastinitis	C135Y	113.2 ± 3.3
UNS38	Brain abscess	V21D	100
UNS35	Brain abscess	R50W	91.9 ± 0.9
UNS40	Liver abscess	[b]–	82.4 ± 26.5
NMH2	Brain abscess	V21D	61.3 ± 2.3
UNS32	Liver abscess	S117I	54.5 ± 3.7
JICC 1063	Liver abscess	V30A, C135Y	53.0 ± 7.5
UNS45	Liver abscess	V30A	46.1 ± 8.6
JICC 40138-2	Infective endocarditis	42Q_44Ldup, C135Y	42.0 ± 2.7
F600	Abdominal abscess	–	48.2 ± 0.5
JICC 33616	Brain abscess	42Q_44Ldup, C135Y	34.6 ± 5.8

[a]Relative hemolytic activity showed ILY hemolytic activity in the culture supernatant of UNS38 set as 1. The data represent the mean values ± standard deviation of 3 replicates each. [b]No amino acid substitution was observed in the amino acid sequence of LacR.

[Modified table from Tomoyasu *et al*., 2013]

Among 57 strains, nine ILY high-producing strains (A4676a, UNS46, UNS38, UNS35, NMH2, JICC 1063, UNS45, 40138-2, and JICC 33616) had a point mutation or an insertion mutation in the

DeoR-type helix-turn-helix domain predicted by the sequence motif search; this domain also appears to be important for DNA binding of LacR (Table 5). These results strongly suggest that mutation in *lacR* is required for the overproduction of ILY, which is associated with an increase in pathogenicity of *S. intermedius*. However, two ILY high-producing strains (UNS40, F600) did not have mutations in the amino acid sequence of LacR or in the *lacR* promoter region and could produce LacR at the wild-type levels (Data not shown). These results indicated that an additional factor(s) besides LacR might also play an important role in regulating *ily* expression.

2 – 3 – 6. Complementation of Δ*lacR* mutant by the mutated *lacR*.

We further examined whether 9 different mutations (R37L, L48F, V2D, R50W, S117I V30A, 42Q_44Ldup, S117N, and C135Y) could affect the function of LacR. Each mutated *lacR* was cloned into pSETN1 and transformed into the Δ*lacR* mutant. The ability of the nine *lacR* mutations to complement the Δ*lacR* mutant was analyzed by examining the relative activities of ILY in the culture supernatant by the hemolysis assay (Fig. 11). Transformation with the mutated *lacR* in the helix-turn-helix domain (R37L, L48F, V2D, R50W, V30A, or 42Q_44Ldup) was not able to complement or only partially complemented the ILY-overproducing phenotype, indicating that this domain is important for LacR function. C135Y was the most frequently observed mutation in LacR and the twelve strains analyzed possessed this mutation. Because transformation by the LacR C135Y-expressing plasmid resulted in a decrease in the level of hemolytic activity to that observed in wild-type *lacR*-transformed cells, LacR C135Y was therefore considered to be functional.

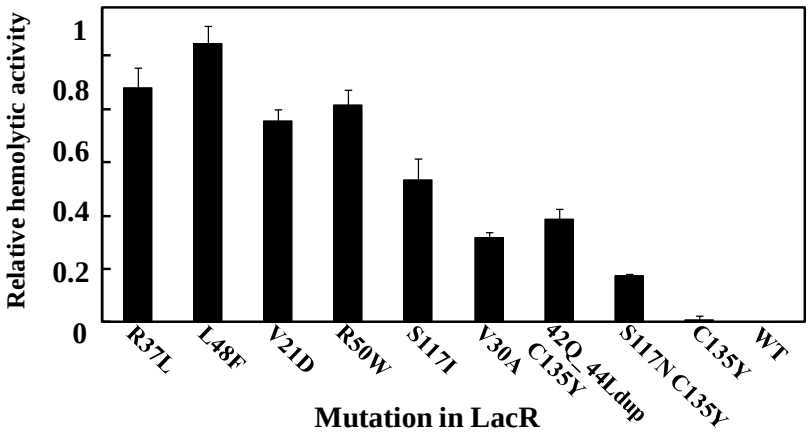

Fig. 11. Complementation of Δ*lacR* mutant by the mutated *lacR*
PC574 Δ*lacR* transformed with plasmids carrying each mutated *lacR* was grown for 48 h at 37° C in MOPS-BHI medium containing 0.1% glucose. Culture supernatant standardized at $OD_{600\ nm}$ was diluted from 25- to 800-fold by 2-fold serial dilutions, and the hemolytic activity was measured. Relative hemolytic activity showed ILY hemolytic activity in the culture supernatant of PC574 Δ*lacR* set as 1. R37L: PC574 Δ*lacR* transformed with p*lacR*(R37L), L48F: with p*lacR*(L48F), V21D: with p*lacR*(V21D), R50W: with p*lacR*(R50W), S117I: with p*lacR*(S117I), V30A: with p*lacR*(V30A), 42Q_44Ldup C135Y: with p*lacR*(42Q_44Ldup, C135Y), S117N C135Y: with p*lacR*(S117N C135Y), C135Y: with p*lacR*(C135Y), and WT: PC574 Δ*lacR* p*lacR*. The data in the graph are the mean ± SD of 6 replicates of independent experiments.

[Tomoyasu *et al.*, 2013]

However, an ILY high-producing strain, JICC 33405 produced LacR C135Y at the levels observed with the wild-type cells (Data not shown). These data suggest that an additional factor(s) to LacR may be involved in the regulation of *ily* expression in strain JICC 33405 as with strains UNS40 and F600.

3. Identification and characterization of MsgA, a novel secreted glycosidase from *Streptococcus intermedius*

3 – 1. Introduction

We showed CcpA and LacR could regulate transcription of *ily* by binding to its promoter region [Tomoyasu *et al.*, 2010, 2013]. These results strongly suggest that the amount and the type of sugar structures in the environment of the bacterial cell are important factors in the pathogenicity of *S. intermedius*. Interestingly, this species has been reported to produce β-galactosidase (β-Gal), *N*-acetyl-β-D-glucosaminidase (β-GlcNAcase), and sialidase (NanA) activities, which is thought to be important for liberating and utilizing monosaccharides from the glycans of glycoproteins [Byers *et al.*, 1999, Paddick *et al*., 2005]. It is also known that *S. intermedius* is the only AGS species that can secrete NanA (neuraminidase), which is encoded by *nanA*. The function of NanA of this species has been analyzed using a *nanA*-deficient strain, and the results show that this enzyme is involved in biofilm degradation and modification of sugar chains on the bacterial cell surface and in the surrounding environment; these changes may influence both bacterium–bacterium and bacterium–host interactions [Takao *et al*., 2010].

Among the AGS species, *S. intermedius* and *S. constellatus* subsp. *pharyngis* exhibit cell-associated β-Gal, β-D-fucosidase (β-Fuc), β-GlcNAcase, and *N*-acetyl-β-D-galactosaminidase (β-GalNAcase) activities [Whiley *et al*., 1999], although the enzyme(s) responsible for these glycosidase activities have not been identified yet. Accordingly, we searched for genes that encode a glycosidase in the *S. intermedius* chromosome.

3 – 2. Materials and Methods

3 – 2 – 1. Bacterial strains and growth conditions.

The bacterial strains used in this study are listed in Table 6. *S. intermedius* PC574 isolated from dental plaque [Nagamune *et al*., 2000] and the derivative strains were cultured at 37°C under anaerobic conditions. Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company, Palo Alto, CA, USA) or 3-(*N*-morpholino)propanesulfonic acid–buffered BHI (MOPS–BHI) broth were used as the culture medium [Tomoyasu *et al.*, 2010]. Antibiotics were added at the following concentrations: chloramphenicol (Cm) at 2 μg/mL, erythromycin (Em) at 1 μg/mL, and spectinomycin (Spc) at 50 μg/mL.

3 – 2 – 2. Databases and sequence alignment.

The nucleotide sequence of *msgA* (SCIM_1144) was obtained from the genome sequence of *S. intermedius* type strain NCDO2227 (GenBank accession No. AP010969). A *msgA* homologue and its pseudogenes from the *S. anginosus* group strains were found using the NCBI Microbial Nucleotide BLAST (National Institutes of Health, USA). Nucleotide sequence alignments between *msgA* from NCDO2227 and its homologues from the AGS strains were performed using the ClustalW program (Kyoto University Bioinformatics Center, Japan; available to http://www.genome.jp/tools/clustalw/). Homologues of MsgA from *S. constellatus* subsp. *pharyngis* C232, C818, C1050, SK1060 were found using NCBI Microbial Protein BLAST. MsgA sequences were analyzed using SignalP 4.1 to predict signal peptide sequences (http://www.cbs.dtu.dk/services/SignalP/), Pfam 27.0 (http://pfam.sanger.ac.uk), the Clusters of Orthologous Groups of proteins (COGs) database (http://www.ncbi.nlm.nih.gov/COG/), and Blocks Search (http://blocks.fhcrc.org/blocks/blocks_search.html) were used to predict the

Table 6: Plasmids and bacterial strains used in this study

Plasmids and Strains	Relevant characteristics	Reference or source
Plasmids		
pSETN1	Streptococci-*Escherichia coli* shuttle vector	Tomoyasu *et al.* 2010
p*lacR*	pSETN1 carrying *lacR* with its own promoter region	Tomoyasu *et al.* 2013
p*msgA*	pSETN1 carrying *msgA* with *lacR* promoter region	This study
pUHE212-1	N-terminally 6× His-tag vector	Gamer *et al.* 1992
pN-his *lacZD*	pUHE212-1 carrying the coding region for LacZ domain of *msgA*	This study
pN-his *hexD*	pUHE212-1 carrying the coding region for GH2 domain of *msgA*	This study
S. intermedius		
NCDO2227	Type strain	Nagamune *et al.* 1992
PC574	ILY low-producing strain from human dental plaque	Nagamune *et al.* 1992
PC574 Δ*msgA*	*msgA* knockout mutant derived from strain PC574	This study
PC574 pSETN1	PC574 transformed with pSETN1	Tomoyasu *et al.* 2013
PC574 Δ*msgA*+pSETN1	PC574 Δ*msgA* transformed with pSETN1	This study
PC574 Δ*msgA*+p*msgA*	PC574 Δ*msgA* transformed with p*msgA*	This study
PC574 Δ*lacR*	*lacR* knockout mutant derived from strain PC574	Tomoyasu *et al.* 2013
PC574 Δ*lacR*+pSETN1	PC574 Δ*lacR* transformed with pSETN1	Tomoyasu *et al.* 2013
PC574 Δ*lacR*+p*lacR*	PC574 Δ*lacR* transformed with pSETN1 *lacR*	Tomoyasu *et al.* 2013
PC574 Δ *lacR* Δ*ily*	*lacR* and *ily* knockout mutant derived from strain PC574	This study
NCDO2227 Δ*nanA*	*nanA* knockout mutant derived from strain NCDO2227	Takao *et al.* 2010
PC574 Δ*nanA*	*nanA* knockout mutant derived from strain PC574	This study

[Imaki *et al*., 2014]

conserved domain architecture in MsgA. Values of pI of both the mature form of MsgA and ILY were predicted using the Compute ProtParam tool (Swiss Institute of Bioinformatics, Switzerland; available at http://web.expasy.org/protparam/).

3 – 2 – 3. Generation of *msgA* and *nanA* knockout mutants from strain PC574.

The *msgA* and the *nanA* knockout mutant (Δ*msgA* and Δ*nanA* mutant) were produced using homologous recombination. In the case of the Δ*msgA* mutant, the 5′ region of *msgA* DNA fragment (1185 bp) was amplified using primer ΔmsgA F and internal primer ΔmsgA EcoRI R (Table 7) and was then digested with *Eco*RI. The 3′ region of the latter (1105 bp) DNA fragment was amplified using the internal primers ΔmsgA SalI F and ΔmsgA R (Table 7), and then digested with *Sal*I. A spectinomycin resistance cassette (Spc cassette) was amplified from the thermosensitive suicide vectors pSET4s [Takamatsu *et al*., 2001] by using primers spc EcoRI F and spc SalI R (Table 7). The *Eco*RI- and *Sal*I-digested Spc cassette was ligated to the *Eco*RI-digested 5′ and *Sal*I-digested 3′ regions, and the ligated fragment was then amplified by PCR with primers ΔmsgA F and ΔmsgA R (Table 7). The amplified fragment was used to construct the Δ*msgA* mutant. To knock out *nanA* that encodes NanA in PC574, the Δ*nanA* region was amplified by PCR with primers ΔnanA F and ΔnanA R (Table 7) from the NCDO2227 Δ*nanA* mutant (Table 6). The resulting 3.7-kbp fragments were recovered and further amplified with primers ΔnanA nested F and ΔnanA nested R (Table 7). The amplified fragment was used to construct the Δ*nanA* mutant. The Δ*msgA* and the Δ*nanA* mutants were produced via transformation of competence-stimulating peptide (CSP)-treated PC574 cells with each PCR amplicon according to the method described previously [Tomoyasu *et al.*, 2010]. Colonies were selected on BHI agar containing 50 μg/mL Spc. Disruption of *msgA* or *nanA* was confirmed using

Table 7: Oligodeoxynucleotides used in this study

Purpose	Name	Sequence (5′ to 3′)
Disruption of *msgA*	ΔmsgA F	GTTGCTTATGTTTTGAACTGTGAGAAAGAC
	ΔmsgA EcoRI R	CTCCGTTTTTTAACCCAGTTGAATTCACAG
	ΔmsgA SalI F	GTGCAAGATGGGTCGACACCTCCATCTACG
	ΔmsgA R	CAAGAAATAGTTCAGATGAATCATGAAACG
	spc EcoRI F	AAACAATGAATTCGTTTACACTTACTTTAG
	spc SalI R	TCTGTCGACCAATTAGAATGAATATTTCCC
Complementation of Δ*msgA* mutant	In-Fusion msgA F	GTGTTACAACTGGTTAACAATTCAATGAAAGG
	In-Fusion msgA R	TTATTTTTTCTTTCGATTTTTCTTGACAAAAAATC
	In-Fusion placR F	CGAAAGAAAAAATAATTAATATTTTAATCACCTGTTCGTGAAGCTGC
	In-Fusion placR R	AACCAGTTGTAACACTTTGTCTCCTTTCTAATCATATAATTAGATTCC
Cloning of coding region for LacZ domain or hexosaminidase domain into 6× His tag vector	LacZ domain F	CGTTGGATCCATGTTTTTATATAGTGTATTTTCAG
	LacZ domain R	GCTGTCCTAGGTTATTTCATAGCAGCTACATCTCC
	Hex. domain F	GAAATTGGATCCATGACAGCGATTGAAACTTTTG
	Hex. domain R	CGTAGCTGCAGGTGTGGATCAATCTTGCAC
Disruption of *ily*	Δily F	CGCCGCCTGACTAACCTTTAAGCGCCTTGC
	Δily EcoRI R	GCTGAATTCGGTGCTGCCGAAGAGAGAACG
	Δily SalI F	CTTCGTCGACAGTTTGAAGATAAAGTTGTG
	Δily R	GAACAGAAGAAGCTTCTGCCTTCTTGGCTG
Disruption of *nanA*	ΔnanA F	CAATCCCTATATAACTTTAAGTGTTTGTTG
	ΔnanA R	GATATCATGTAGAGAAACAGAAAAAACTAC
	ΔnanA nested F	TGAGAGAGGAGGGATTTTCTTACTGATCGG
	ΔnanA nested R	CTAGAAGATACACTTCTGGGATAAATAGGG
Probes for qRT-PCR	qRT-msgA F	CTACACCCAAAGTGGTGAAGCAGAGAGTGG
	qRT-msgA R	CGATAAATACCACTACCAGAATACCAGCGC

[Imaki *et al*., 2014]

PCR and by disappearance of enzymatic activity of MsgA or NanA (Table 8).

3 – 2 – 4. Complementation of *S. intermedius* PC574 Δ*msgA* strain.

To construct the MsgA-producing plasmid, a Streptococci–*E. coli* shuttle vector pSETN1-derived plasmid (p*lacR*), in which cloned a *lacR* fragment containing the putative native promoter was used [Tomoyasu *et al*., 2013]. The *msgA* fragments were amplified by PCR using the primers In-Fusion msgA F and In-Fusion msgA R from the chromosomal DNA of *S. intermedius* type strain NCDO2227. The p*lacR* was linearized by inverse PCR using primers In-Fusion placR F and In-Fusion placR R (Table 7). The linearized plasmid contained pSETN1, the putative native promoter of *lacR*, and 15 bp extensions (5') complementary to the ends of *msgA*. The *msgA* fragments were cloned within the linearized plasmid using the In-Fusion® HD Cloning Kit (Takara Bio Inc., Tokyo, Japan) as described in the user manual. The In-Fusion reaction was carried out for 15 min at 50°C, then placed on ice. *E. coli* DH5αZ1 was transformed by the resultant plasmid (p*msgA*). Subsequently, the p*msgA* was extracted and used for transformation of a CSP-treated PC574 Δ*lacR* mutant. Transformants were selected and isolated on a BHI agar plate containing 2μg/ml Cm and 50μg/ml Spc.

3 – 2 – 5. Preparation of an *ily* knockout in the PC574 Δ*lacR* mutant.

The 5′ region of the *ily* DNA fragment (839 bp) was amplified using primer Δily F and internal primer Δily EcoRI R (Table 7) and was then digested with *Eco*RI. The 3′ region of the latter (929 bp) DNA fragment was amplified using internal primers Δily SalI F and Δily R (Table 7) and then digested with *Sal*I. The *Eco*RI- and *Sal*I-digested Spc cassette was ligated to the *Eco*RI-digested 5′ region and *Sal*I- digested 3′ region of *ily*, and the ligated fragment was then amplified with primers Δily F and Δily R (Table 7). The amplified fragment was used to generate the Δ*ily* mutant. The

Δ*ily* mutant was created by transforming CSP-treated PC574 Δ*lacR* cells (Table 6) with the PCR amplicon, according to the method described previously [Tomoyasu *et al*., 2010]. Colonies were selected on BHI agar containing 50 μg/mL Spc and 1 μg/mL Em. Disruption of *ily* was confirmed using PCR and by checking for the absence of hemolytic activity of PC574 Δ*lacR* [Tomoyasu *et al*., 2013].

3 – 2 – 6. Preparation of glycosidase substrates

Cell- and supernatant-associated glycosidase activity of *S. intermedius*, the culture supernatant, and the purified MsgA was measured using the fluorogenic (4-methylumbelliferyl–linked: 4-MU) substrates: 4-MU-β-D-galactopyranoside, 4-MU-β-D-fucoside, 4-MU-*N*-acetyl-β-D-glucosaminide, 4-MU-*N*-acetyl-β-D-galactosaminide, and 2'-(4-MU)-α-D-*N*-acetylneuraminic acid sodium salt hydrate. All substrates were obtained from Sigma–Aldrich Corporation (St. Louis, MO, USA) and solubilized in dimethyl sulfoxide.

3 – 2 – 7. Purification of MsgA

PC574 Δ*lacR* Δ*ily* double mutant was cultured in 1.0 L of the BHI medium. The culture supernatant and the cells were separated by centrifugation (5000 × *g*). Proteins in the culture supernatant were precipitated by addition of ammonium sulfate to 80% saturation and subsequent centrifugation (10000 × *g*). The precipitated proteins were dissolved in 40 mL of 50 mM sodium acetate buffer (pH 5.5) containing 100 mM NaCl and 0.01% NP-40 and then dialyzed against the same buffer. The harvested cells were resuspended in 5 mL of 50 mM sodium acetate buffer (pH 5.5) containing 1.0 M NaCl and 0.01% NP-40 to extract cell associated MsgA; then, the cells were removed by centrifugation (5000 × *g*). An aliquot of the extracted MsgA was diluted 10-fold with 50 mM sodium acetate buffer (pH 5.5) containing 0.01% NP-40. The resultant dialyzed solution

(from the culture supernatant) or MsgA extracted from the cells was loaded onto a HiTrap SP HP Column (GE Healthcare, Buckinghamshire, UK). Proteins bound to the column was eluted with a linear gradient of 0–1.0 M NaCl in sodium acetate buffer (pH 5.5) containing 0.01% NP-40. Peak fractions of MsgA were identified and combined to measure β-Gal activity by using 4-MU-β-D-galactopyranoside as a substrate. The fraction was further purified using a Superdex 200 (GE Healthcare) gel filtration column pre-equilibrated with 50 mM sodium acetate buffer (pH 5.5) containing 1.0 M NaCl and 0.01% NP-40. Purified MsgA was stored at −80°C until use.

3 – 2 – 8. Protein quantification of MsgA

The concentration of MsgA was determined using a protein molecular weight marker XL-Ladder High (APRO life Science Institute, Inc., Tokushima, Japan). MsgA and the diluted XL-Ladder High (2-fold serial dilutions) were analyzed using an 8% SDS-PAGE gel [Laemmli. 1970] and stained with ORIOLE Fluorescent Gel stain (Bio-Rad Co., CA, USA). The stained proteins were detected and digitized using an ImageQuant LAS 4000 mini (GE Healthcare). The amount of MsgA was calculated using a standard curve with the 250 kD protein (150, 75, 32.5, and 16.3 ng/gel) in XL-Ladder High by using Multi Gauge Ver. 3.0 software (GE Healthcare).

3 – 2 – 9. Preparation of His-tagged recombinant LacZ and GH20 domains of MsgA

The coding region of the LacZ domain (*lacZD*) and GH20 domain (*hexD*) in *msgA* were amplified from the chromosomal DNA of *S. intermedius* type strain NCDO2227. This was done using the primers LacZ domain F and LacZ domain R or Hex. domain F and Hex. domain R (Table 7). The amplified *lacZD* fragment was digested with *Bam*HI and *Avr*II and the amplified *hexD* fragment was digested with *Bam*HI and *Pst*I and cloned into pUHE212-1. *E. coli* DH5αZ1 was transformed by each resultant plasmid (pN-his *lacZD* or pN-his *hexD*). Hyper-expression of

the His- tagged LacZ domain (LacZD) or His-tagged HexD domain (HexD) was induced by adding 1 mM IPTG to *E. coli* cells in mid-exponential phase and incubation continued at 37°C for 2 h. The cells were then harvested by centrifugation (5,000 × *g*) and resuspended in 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 20% sucrose, and 1 mg/ml lysozyme. The suspension was sonicated with an Astrason Ultrasonic Processor and diluted 5-fold with 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$. The resultant cell extract was centrifuged at 10,000 × *g* for 20 min to remove the debris and unbroken cells. The supernatant was loaded onto a nickel affinity column (HisTrap FF 5mL: GE Healthcare) equilibrated with buffer A (20 mM Tris-HCl pH 8.0, 300 mM NaCl, and 20 mM imidazole). Each His-tagged protein was eluted with a linear gradient of 20–500 mM imidazole in buffer A. Peak fractions of LacZD were diluted 10-fold with buffer B (50 mM sodium acetate buffer pH 6.0, 0.01% NP-40) and peak fractions of HexD were diluted 10-fold with buffer C (50 mM sodium acetate buffer pH 5.5, 0.01% NP-40). Each diluted sample was loaded onto a HiTrap SP HP Column (GE Healthcare). LacZD was eluted with a linear gradient of 0–1.0 M of NaCl in buffer B and HexD was eluted with a linear gradient of 0–1.0 M of NaCl in buffer C. Purified proteins were stored at −80°C until use.

3 – 2 – 10. Detection of glycosidase activity in cell suspensions and culture supernatants

S. intermedius PC574 and its derivative strains were cultured in MOPS–BHI medium at 37°C for 24 h. Cells were separated from the culture supernatants by centrifugation (5000 × *g*). Each cell suspension (optical density at 600 nm OD_{600} = 1.0) was prepared in ice-cold 20 mM Tris-HCl buffer (pH 7.5). Assays employing fluorogenic substrates were performed as described previously with minor modifications [Homer *et al.*, 1994]. An assay mixture (100 μL) containing 70 mM citrate buffer (pH 5.5), 250 μM 4-MU–linked substrate, and 10 μL of each cell suspension or

culture supernatant was incubated at 37°C. Each aliquot (10 µL) of the reaction mixture was taken at suitable time points and mixed with 190 µL of 0.5 M sodium carbonate buffer (pH 10.2) to terminate the enzymatic reaction. All assays were set up in triplicate, and the release of 4-MU was quantified on the microplate reader Infinite M200 (Tecan Group Ltd., Männedorf, Switzerland); excitation and emission wavelengths used were 380 nm and 460 nm, respectively.

3 – 2 – 11. Specific glycosidase activity

Glycosidase activities of purified LacZ and GH20 domains of MsgA and intact MsgA were expressed as units/nM. Units are defined as the production of 100 nM 4-MU/min from 250 µM 4-MU–linked substrate in 70 mM citrate buffer (pH 5.5) at 37°C.

3 – 2 – 12. Kinetic parameters and the optimum reaction temperature of MsgA

The kinetic parameters, Michaelis constants (*K*m) and *V*max for β-Gal, β-Fuc, β-GlcNAcase, and β-GalNAcase activities were determined as described previously with minor modifications [Homer *et al.*, 1994]. An assay mixture (100 µL) contained 70 mM citrate buffer (pH 5.5), 1 nM MsgA, and variable concentrations of a 4-MU–linked substrate. The concentration of substrates varied from 20 to 1000 µM in case of β-Gal, from 20 to 500 µM in the case of β-GalNAcase, from 10 to 250 µM in the case of β-GlcNAcase, and from 100 to 2500 µM in the case of β-Fuc, respectively; all reaction mixtures were incubated at 37°C and set up in duplicate. A 10 µL aliquot of each reaction mixture was removed at suitable time points and mixed with 190 µL of 0.5 M sodium carbonate buffer (pH 10.2) to terminate the enzymatic reaction. Concentrations of released 4-MU were calculated by comparing fluorescence values with those obtained from standard concentrations of 4-MU. Optimum temperatures of MsgA enzymatic activities were determined using 250 µM 4-MU–linked substrates and 1 nM MsgA to assess the amount of released 4-MU

under several temperatures.

3 – 2 – 13. Detection of glycans on α_1-antitrypsin by periodic acid–Schiff staining

PC574, Δ*msgA*, and Δ*nanA* mutants were cultured until the stationary phase in the BHI medium for 24 h. Culture supernatants were separated by centrifugation. A solution of 10 mg/mL α_1-antitrypsin from human plasma (α1AT; Sigma–Aldrich Corporation) was diluted 10-fold with culture supernatant in the presence or absence of 2 nM purified MsgA and incubated for 0, 24, and 48 h. After incubation, 1 μg of α1AT was analyzed on a 12% SDS-PAGE gel. For periodic acid–Schiff (PAS) staining, the gel-resolved proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). PAS staining was performed as described previously with minor modifications [Thornton *et al*., 1996]. Briefly, the blotted PVDF membrane was rinsed in deionized water and then incubated with 1.0% periodic acid in 3% acetic acid for 30 min. The periodic acid solution was removed and the membrane was rinsed in deionized water twice. Schiff's reagent (Merck KGaA, Darmstadt, Germany) was added and incubated for 15 min. Reddish pink bands of stained glycoproteins would then be visible. Schiff's reagent and background staining were removed by washing several times with a destaining solution (0.1% sodium bisulfite in 10 mM HCl). The destained membrane was rinsed in deionized water and then dried.

3 – 2 – 14. Quantitative RT-PCR analysis

PC574, the PC574 Δ*lacR* mutant containing control vector pSETN1, and the PC574 Δ*lacR* mutant complemented with a LacR-producing plasmid p*lacR* (Table 6) were cultured in the MOPS–BHI medium at 37°C for 24 h under anaerobic conditions, and the cells were subsequently separated by centrifugation (5000 × *g*). Isolation of total RNA from the cells and quantitative RT-PCR

(qRT-PCR) analysis were performed as described previously [Tomoyasu *et al.*, 2010]. The primer set qRT-msgA F and qRT-msgA R (Table 7) was used for quantification of *msgA* mRNA.

3 – 3. Results

3 – 3 – 1. Bioinformatic analysis of *msgA*.

We found an open reading frame (ORF) in the *lac* operon which seemed to encode a large glycosidase (MsgA) with 2235 amino acids. A homology search and prediction of conserved domains of MsgA demonstrated that this protein was homologous to the glycosidases that contain a glycosyl hydrolase family (GH) 2 and a GH 20 catalytic domains [Henrissat & Davies. 1997]. It is known that glycosidases such as β-Gal and β-D-mannosidase belong to GH2, which includes *E. coli* β-Gal (LacZ). Glycosidases, that have the GH20 catalytic domain and can remove β1,4-linked *N*-acetyl-D-hexosamine residues from nonreducing ends of *N*-acetyl-β-D-glucosamine or *N*-acetyl-β-D-galactosamine residues of oligosaccharides, have been previously reported [Intra *et al*., 2008, Slámová *et al*., 2010].

3 – 3 – 2. Characterization of the Δ*msgA* mutant.

In order to confirm that the *msgA* mutation affects cell- and supernatant-associated glycosidase activities, an *msgA* knockout mutation was introduced into the PC574 genome. In the Δ*msgA* mutant, the four relevant cell- and supernatant-associated glycosidase activities were significantly reduced (Table 8, Data not shown). However, cell-associated NanA was not affected (Table 8). To exclude the possibility that the mutant phenotypes resulted from other mutations in the chromosome, the Δ*msgA* mutation was complemented *in trans* with a recombinant plasmid (p*msgA*) carrying the *msgA* and *lacR* promoter region of *S. intermedius*. The levels of the four glycosidase activities that were lost by the Δ*msgA* mutation were restored and increased by approximately two-fold by p*msgA* complementation compared to the wild-type strain (Table 8).

These results strongly indicate that MsgA is a multi-substrate glycosidase that has β-Gal, β-Fuc, β-GlcNAcase, and β-GalNAcase activities.

Table 8: Relative glycosidase activity

	Relative activity: mean (SD)			
	Δ*msgA*[a]	Δ*msgA*+ p*msgA*[b, c]	Δ*lacR*[a]	Δ*nanA*[a]
β-Gal	<0.01	1.8 (0.20)	4.6 (0.23)	1.1 (0.06)
β-Fuc	<0.01	2.0 (0.04)	5.5 (0.23)	1.3 (0.01)
β-GlcNAcase	<0.01	2.2 (0.15)	6.3 (0.45)	1.2 (0.01)
β-GalNAcase	<0.01	2.2 (0.21)	5.0 (0.27)	1.1 (0.06)
NanA	1.1 (0.06)	–[d]	0.5 (0.09)	<0.01

Glycosidase activity from 10 μL cell suspension (OD_{600} = 1.0) of wild-type cells ([a]PC574 or [b]PC574 pSETN1) set as 1. [c] Δ*msgA*+p*msgA*: PC574 Δ*msgA* p*msgA*. [d] –: Not determined.

[Imaki *et al*., 2014]

Because *msgA* localizes in the *lac* operon, its transcriptional activity is likely to be under the control by LacR, and expression of this gene might be enhanced by a Δ*lacR* mutation. Our results supported the supposition that four cell-associated glycosidase activities of the Δ*lacR* mutant were increased by approximately 5- to 6-fold (Table 8). A slightly weaker cell-associated NanA activity and a stronger α-Glu activity (compared to the parental strain) were observed of Δ*lacR* mutant. Therefore LacR appears to control the expression of these glycosidases and consequently the Δ*lacR* mutation may affect their expression and activity. Thereafter, we examined the amount of mRNA from *msgA* in PC574, the Δ*lacR* mutant and the complemented strain of Δ*lacR* mutant by measuring the relative amounts of *msgA* mRNA (*msgA*/*gyrB*) in these strains (Fig. 12). The expression level of *msgA* in the Δ*lacR* cells was 40.4-fold higher than that in PC574 and reverted to

a normal level as in PC574 after p*lacR* complementation. In addition, the levels of the four glycosidase activities that were elevated by the Δ*lacR* mutation decreased to the level of the wild-type strain after p*lacR* complementation (Data not shown). These results indicate that LacR controls *msgA* expression in the *lac* operon.

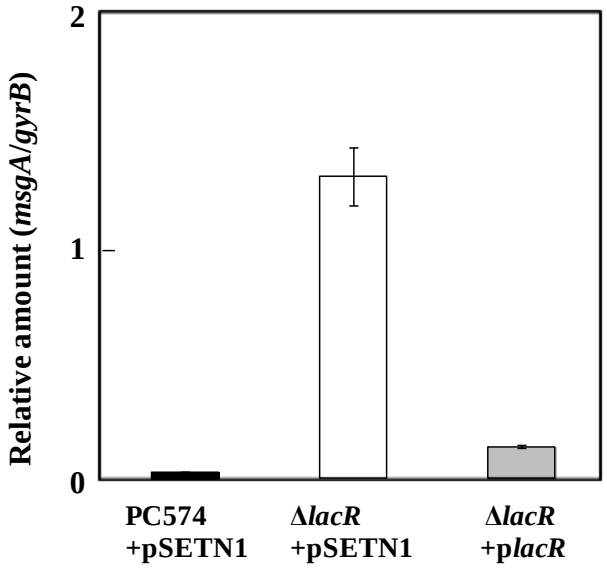

Fig. 12. *msgA* transcriptional activity in the Δ*lacR* mutant. A wild-type strain, its Δ*lacR* mutant, and its *lacR*-complementation strain were grown for 24 h at 37° C in the MOPS–BHI medium. The expression levels of *msgA* in PC574+pSETN1, the Δ*lacR*+pSETN1, and Δ*lacR*+p*lacR* are indicated relative to the *gyrB* expression level. The results are plotted on a logarithmic scale in the vertical axis. The data are shown as a mean and SD of 6 replicates.

[Imaki *et al*., 2014]

3 – 3 – 3. Purification of MsgA.

Because a stronger glycosidase activity possibly related to MsgA was observed in the Δ*lacR* mutant, MsgA was purified from this mutant. The Δ*lacR* mutant could produce a larger amount of ILY [Tomoyasu *et al*., 2013] and the predicted pI values of MsgA and ILY were similar: 9.17 and 9.48, respectively. Indeed, MsgA and ILY were co-purified using cation exchange chromatography (Data

not shown); accordingly, the Δ*lacR* Δ*ily* double mutant was used for purification. MsgA was purified using cation exchange followed by Superdex 200 gel filtration chromatography.

3 – 3 – 4. Purification and characterization of LacZ and GH20 domains of MsgA.

MsgA has two predicted catalytic domains (LacZ and GH20 domain) responsible for its glycosidase activity and these domains appear to exhibit different activities. Therefore, we purified the truncated MsgA, which contained either the LacZ domain (LacZD) or the GH20 domain (HexD) and determined the specific glycosidase activities of each domain (Table 9). Purified LacZD exhibited weaker activity than MsgA with as little as 10% β-Gal and 36% β-Fuc activities.

Table 9: Specific glycosidase activities[a] of MsgA and truncated MsgA.

	Relative activity: mean (SD)		
	MsgA	LacZD	HexD
β-Gal	28.3 (2.79)	2.9 (0.05)	<0.01
β-Fuc	2.2 (0.31)	0.8 (0.11)	<0.01
β-GlcNAcase	44.2 (6.32)	0.4 (0.06)	47.3 (3.40)
β-GalNAcase	11.4 (1.63)	0.1 (0.02)	13.4 (1.43)

[a] Specific glycosidase activities of purified proteins were expressed as units/nM protein.

[Imaki *et al*., 2014]

The domain downstream of our truncated region (*e.g.* Big4) might require the correct folding and/or stabilization of LacZD. In addition, slight but significant β-GlcNAcase and β-GalNAcase activities were also observed, although these were less than 0.1% the activity of MsgA. HexD

exhibited both β-GlcNAcase and β-GalNAcase activities and had comparable enzymatic activity to MsgA. In contrast to LacZD, this domain did not exhibit any β-Gal and β-Fuc activities. These results strengthen our hypothesis that β-Gal, β-Fuc, β-GlcNAcase, and β-GalNAcase activities are derived from MsgA.

3 – 3 – 5. Enzymatic parameter of purified MsgA.

The optimal pH for each glycosidase activity in crude cell-free extracts from *S. intermedius* was previously reported to be 5.5-6.0 for β-Gal and 5.0-5.5 for both of β-GlcNAcase, and β-GalNAcase, respectively [Homer *et al*., 1994]. Accordingly, the enzymatic parameters of these glycosidases have been analyzed and reported at pH 5.5. We also investigated the optimal pH for MsgA glycosidase activities using purified enzyme and confirmed that those for β-Gal and for β-GlcNAcase were around 6.0 and 5.0, respectively (Data not shown). Consequently, the kinetic parameters and the optimal reaction temperatures of purified MsgA were measured using a 0.1 nM enzyme solution at pH 5.5. The initial rate of reaction of MsgA with the fluorogenic substrates was measured at various temperatures and concentrations of the substrates. K_m and V_{max} with the 4 substrates were determined using Lineweaver–Burk plots, and k_{cat} was calculated (Table 10). K_m values of β-Gal, β-GlcNAcase, and β-GalNAcase showed a similar tendency, which was previously reported for a cell suspension [Homer *et al*., 1994]. MsgA showed the lowest K_m with 4-MU-*N*-acetyl-β-D-glucosaminide and the highest k_{cat} with 4-MU-β-D-galactopyranoside. Because this enzyme showed the highest K_m value with 4-MU-β-D-fucoside and the lowest k_{cat} with 4-MU-*N*-acetyl-β-D-galactosaminide compared to the other enzymatic activities, these two activities do not seem to be the main function of MsgA. Therefore, the primary function of MsgA *in vivo* seems to be the removal of *N*-acetyl-β-D-glucosamine and galactose residues from

oligosaccharide chains such as N- and O-linked glycans.

Table 10. Kinetic parameters of purified MsgA with fluorogenic substrates

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
4-MU-β-D-galactopyranoside	0.548	244.7	446.5
4-MU-β-D-fucoside	2.613	64.2	24.6
4-MU-*N*-acetyl-β-D-glucosaminide	0.024	134.2	5591.7
4-MU-*N*-acetyl-β-D-galactosaminide	0.144	46.3	321.5

Assays were performed in 70 mM citrate buffer, pH 5.5, at 37°C, using 1 nM MsgA.

[Imaki *et al*., 2014]

The effect of temperature on enzymatic activities of MsgA was also assessed (Fig. 13). β-Gal and β-Fuc activities are thermolabile: the optimal temperature was 40°C. In contrast, β-GlcNAcase and β-GalNAcase activities are thermostable: the optimum temperatures were 58°C and 55°C, respectively.

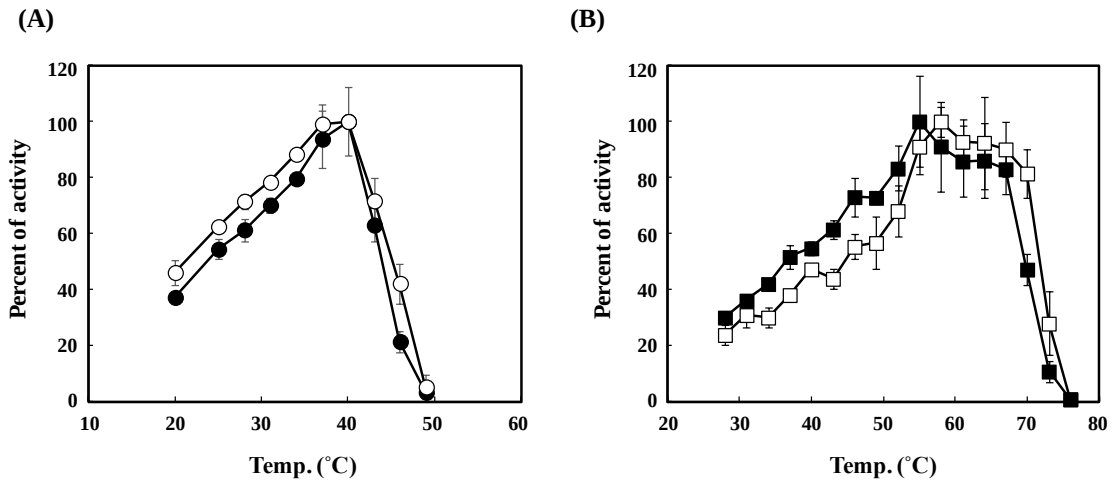

Fig. 13. The optimal temperature for enzymatic activities of MsgA. Enzymatic activity is shown as a percentage of the maximum for each fluorogenic substrate. (A) β-Gal (open circles) and β-Fuc (closed circles) activities. (B) β-GlcNAcase (open squares) and β-GalNAcase (closed squares) activities. The data are shown as a mean and SD of 3 replicates.

[Imaki *et al*., 2014]

3 – 3 – 6. Degradation of sugar chains on human α1AT.

It has been shown that glycosidase activities of *S. intermedius* such as NanA, β-Gal, and β-GlcNAcase are necessary for procurement and utilization of monosaccharides, sialic acid, *N*-acetyl-β-D-glucosamine, and galactose respectively, which are constituents of carbohydrate side chains of glycoproteins [Byers *et al*., 1999]. Our data indicated that MsgA participates in degradation of glycans. Therefore, we analyzed the degradation of glycans by MsgA using human α1AT, a protein abundantly present in plasma and containing biantennary complex-type N-glycans [Nakagawa *et al.*, 2006]. Because a significant amount of MsgA (ca. 20% of total activity) and NanA activity (ca. 41% of total activity) from wild-type cells existed in the culture supernatant under our experimental conditions, α1AT was incubated with the culture supernatant of PC574, Δ*msgA*, and Δ*nanA* mutants at 37°C (Fig. 14A).

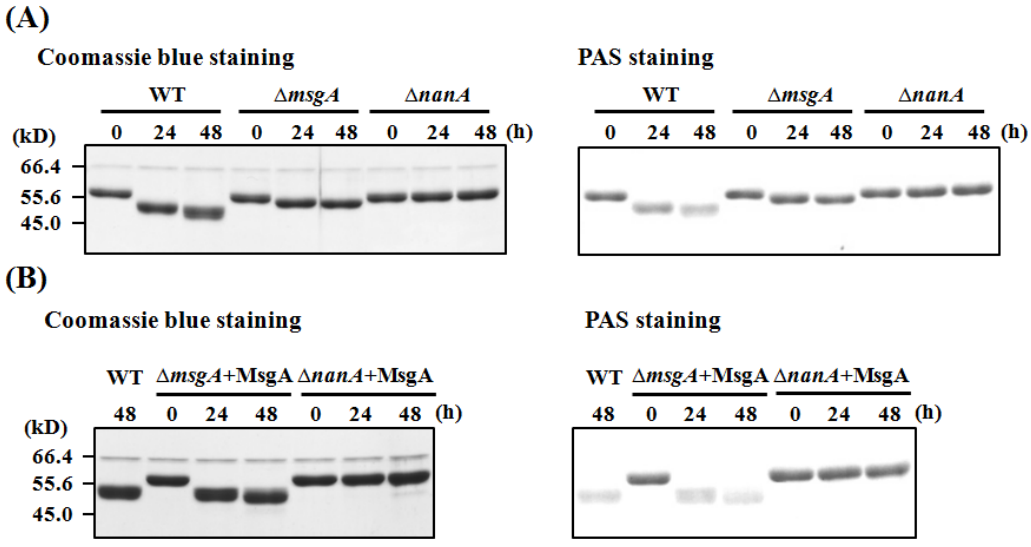

Fig. 14. Comparison of molecular mass and the amount of α_1AT glycans after incubation with culture supernatant from the Δ*msgA* mutant, the Δ*nanA* mutant, and the wild-type (WT) strain. α1AT (1 mg/mL) was incubated with each culture supernatant (A) or culture supernatant containing 2 nM purified MsgA for the indicated time periods (B). WT: culture supernatant from PC574, Δ*msgA*: from PC574 Δ*msgA*, Δ*nanA*: from PC574 Δ*nanA*, Δ*msgA*+MsgA: from PC574 Δ*msgA* in the presence MsgA, Δ*nanA*+MsgA: from PC574 Δ*nanA* in the presence MsgA.

[Imaki *et al*., 2014]

The molecular mass of α1AT was gradually reduced when the protein was incubated with the culture supernatant from the PC574 strain, and an approximately 5.9 kD and 8.2 kD reduction was observed after 24 h and 48 h of incubation, respectively. Because the smaller size of α1AT corresponded to the bands with significant reduction in the signal intensity of PAS staining, the glycosidases produced by *S. intermedius* seem to remove N-glycosylated sugar chains. Only a marginal reduction in molecular mass (ca. 1.8 kD) of α1AT was observed after incubation with the Δ*msgA* culture supernatant. The reduction of molecular mass of α1AT was not observed after incubation with the Δ*nanA* culture supernatant.

We also analyzed whether purified MsgA could remove N-glycosylated sugar chains of α1AT. The results, shown in Fig. 14A, suggest that NanA activity is necessary for the degradation of these sugar chains. Therefore, purified MsgA was supplemented in the culture supernatant from Δ*msgA* mutants, which contained intrinsic NanA activity (Fig. 14B). MsgA-supplemented culture supernatant could reduce the molecular mass of α1AT and the signal intensity of PAS staining to the wild-type level. In addition, MsgA-supplemented culture supernatant from the Δ*nanA* mutant did not exhibit any molecular mass reduction or signal intensity of PAS staining. These results strongly suggest that MsgA has exoglycosidase activities and the NanA activity to remove sialic acid residues is required to initiate the degradation of glycans.

CHAPTER Ⅲ

Conclusion

It has been reported that the genes involved in basic metabolic processes, such as catabolism of complex carbohydrates, are crucial to the pathogenicity of many streptococci [Graham *et al*., 2006, Orihuela *et al*., 2004, Shelburne *et al*., 2008a, b]. Therefore, transcriptional repressor for genes of sugar metabolism such as CcpA and LacR were believed to have an important role in regulation of the pathogenicity of streptococci. Indeed, CcpA and LacR could control the levels of *ily* expression in *S. intermedius* [Tomoyasu *et al*., 2010, 2013]. CcpA can regulate *ily* expression (Fig. 2) and growth rate, depending on the extracellular glucose or utilizable carbohydrate concentration such as glucose. LacR also regulates the levels of ILY to monitor the amount of extracellular galactose (Fig. 9) and disruption of *lacR* causes constitutive overproduction of ILY (Fig. 8A, B) and consequently an increase in cytotoxicity against the HepG2 (Fig. 10). In addition, we also showed ILY-overproducing strains isolated from deep-seated abscesses such as brain and liver abscesses have a loss-of-function mutation in LacR (Table 5, Fig. 11). These results strongly suggest that the amount and the type of sugar structures in the environment of the bacterial cell are important factors in the pathogenicity of *S. intermedius*. Meanwhile, the pathogenicity of several Gram-negative pathogenic bacteria including *Salmonella enterica* serovar Typhimurium, enterohemorrhagic *E. coli*, and *Yersinia pestis* can be regulated by the stress-inducible chaperone (DnaK) and the stress-inducible proteases (ClpXP, Lon) [Iyoda & Watanabe. 2005, Jackson *et al*. 2004, Takaya *et al.* 2002, 2004, Tomoyasu *et al.* 2005, Yamamoto *et al.* 2001]. Therefore, these bacteria are believed to control the expression of virulence factors and possess the ability to sense stresses such as those

presented by an effective immune system and accompanying fever in the host organism. Therefore we investigated whether such the stress-inducible proteins participated in the expression control of virulence factors of *S. intermedius* as well as in the Gram-negative bacteria. *S. intermedius dnaK* null mutation and *clpP* (encodes catalytic subunit of ClpXP protease) null mutation did not cause reduction of the levels of pathogenic factors such as in the activity of ILY and hyaluronidase in the culture medium, compared to the wild-type strain [Tomoyasu *et al.* 2012, Data not shown]. Thus, different from Gram-negative bacteria, the expression of virulence factors and cytotoxicity of *S. intermedius* seemed to be mainly regulated by the amount of extracellular utilizable carbohydrates rather than stresses in the host organism (immune system and fever). Since *S. intermedius* normally presents in the human oral cavity, this bacterium requires creating the mechanisms to utilize nutrients such as sugar surrounding the cells to survive in normal habitat. Therefore, ILY and MsgA might control the amount and the type of sugar in the environment rather than stresses in the host organism. *S. intermedius* exhibits cell-associated NanA, β-Gal, β-GlcNAcase activities [Homer *et al*., 1994]. It has been showed that the microbiota that includes *S. intermedius*, which produces these activities could survive under dental restoration conditions by extracting sugars from serum glycoproteins coming from the pulp through a patient's dental tubules to the infected dentine [Paddick *et al.*, 2005]. This finding supports our idea that MsgA and NanA have a crucial function in the normal habitat of *S. intermedius*, such as the human oral cavity. MsgA showed a weak β-Fuc activity; nevertheless, it does not belong to the GH1 or GH30 which also has the β-Fuc activity (the CAZy database: http://www.cazy.org/Glycoside-Hydrolases.html). It has been reported that *E. coli* LacZ, which has a GH2 domain, recognizes and catalyzes a reaction with β-D-fucosyl moieties weakly [Parikh & Matsumura. 2005, Zhang *et al.*, 1997]. The β-Gal activity of MsgA has a lower K_m

value with 4-MU-β-D-galactoside, but it showed the highest k_{cat} among four glycosidase activities of MsgA (Table 10). Therefore, substrate recognition of MsgA's GH2 catalytic domain seems to be loose, and this might be the reason for the weak β-Fuc activity. In addition, the optimum temperature for β-Gal and β-Fuc activity is 40℃, which is different from optimum temperatures of the other two glycosidase activities, which were approximately 55℃ (Data not shown). These data also support our idea that the β-Fuc activity is derived from the GH2 catalytic domain of MsgA. The *msgA* gene is located in the *lac* operon and regulated by LacR (Table 8, Fig. 12). Because many N- and O-linked glycans contain β-D-galactosyl moieties, MsgA may cut off the galactose cooperating with NanA (Fig. 14). The resulting galactose might inactivate LacR, and this change should upregulate production of MsgA and ILY. Therefore, it is considered that MsgA and NanA not only play a role in the procurement of utilizable carbohydrates in the normal habitat (and in colonized deep-seated organs such as liver and brain) but also control pathogenicity of *S. intermedius* by regulating *ily* expression. Functions of surface-associated exoglycosidases: NanA, β-Gal (BgaA), and β-GlcNAcase (StrH) have been characterized using *S. pneumoniae* [Banerjee *et al.*, 2010, Brittan *et al.*, 2012, Burnaugh *et al.*, 2008, Dalia *et al.*, 2010, Kadioglu *et al.*, 2008, Limoli *et al.*, 2011]. It is believed that the functions of these glycosidases include detachment of sugars from oligosaccharides to obtain nutrients, as well as participation in pneumococcal colonization and pathogenesis of the disease by deglycosylating human glycoconjugates in order to unmask receptors for adherence and to avoid opsonophagocytic killing by human neutrophils [Kadioglu *et al.*, 2008, Dalia *et al.*, 2010]. Because glycosidases such as in MsgA of *S. intermedius* might also play a similar role in the pathogenesis, further research on their involvement in infectivity, cytotoxicity toward cultured human cells, and evasion of phagocytic killing seems to be worthwhile.

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List of publications

I. **Hidenori Imaki**, **Toshifumi Tomoyasu, Naoki Yamamoto, Chiharu Taue, Sachiko Masuda, Atsushi Tabata, Robert A. Whiley, Hideaki Nagamune.** (2014) Identification and Characterization of a Novel Secreted Glycosidase, with Multiple Glycosidase Activities, in *Streptococcus intermedius*. *J Bacteriol*. **196:**2817-2826.

II. **Toshifumi Tomoyasu, Hidenori Imaki, Sachiko Masuda, Ayumi Okamoto, Hyejin Kim, Richard D. Waite, Robert A. Whiley, Ken Kikuchi, Keiichi Hiramatsu, Atsushi Tabata, Hideaki Nagamune.** (2013) LacR Mutations Are Frequently Observed in *Streptococcus intermedius* and Are Responsible for Increased Intermedilysin Production and Virulence. *Infect. Immun.* **81:**3276-3286. (T.T. and H.I. contributed equally to this work)

III. **Toshifumi Tomoyasu, Atsushi Tabata, Hidenori Imaki, Keigo Tsuruno, Aya Miyazaki, Kenji Sonomoto, Robert Alan Whiley, Hideaki Nagamune.** (2012) Role of *Streptococcus intermedius* DnaK chaperone system in stress tolerance and pathogenicity. *Cell Stress Chaperones* **17:**41-55.

IV. **Toshifumi Tomoyasu, Atsushi Tabata, Riki Hiroshima, Hidenori Imaki, Sachiko Masuda, Robert A. Whiley, Joseph Aduse-Opoku, Ken Kikuchi, Keiichi Hiramatsu, and Hideaki Nagamune.** (2010) Role of Catabolite Control Protein A in the Regulation of Intermedilysin Production by *Streptococcus intermedius*. *Infect. Immun.* **78:**4012-4021.