LacR Mutations Are Frequently Observed in *Streptococcus intermedius* and Are Responsible for Increased Intermedilysin Production and Virulence

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Streptococcus intermedius secretes a human-specific cytolyisin, intermedilysin (ILY), which is considered to be the major virulence factor of this pathogen. We screened for a repressor of *ily* expression by using random gene disruption in a low-ILY-producing strain (PC574). Three independent high-ILY-producing colonies that had plasmid insertions within a gene that has high homology to *lacR* were isolated. Validation of these observations was achieved through disruption of *lacR* in strain PC574 with an erythromycin cassette, which also led to higher hemolytic activity, increased transcription of *ily*, and higher cytotoxicity against HepG2 cells, compared to the parental strain. The direct binding of LacR within the *ily* promoter region was shown by a biotinylated DNA probe pulldown assay, and the amount of ILY secreted into the culture supernatant by PC574 cells was increased by adding lactose or galactose to the medium as a carbon source. Furthermore, we examined *lacR* nucleotide sequences and the hemolytic activity of 50 strains isolated from clinical infections and 7 strains isolated from dental plaque. Of the 50 strains isolated from infections, 13 showed high ILY production, 11 of these 13 strains had one or more point mutations and/or an insertion mutation in LacR, and almost all mutations were associated with a marked decline in LacR function. 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*.

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. *S. intermedius* belongs to the Anginosus group of streptococci (AGS), which also includes *Streptococcus anginosus* and *Streptococcus constellatus* (1, 2). AGS tend to form local suppurative infections, and these organisms are the most common pathogens associated with bacterial intracerebral abscesses (1–6). *S. intermedius* is the most pathogenic species of AGS and a leading cause of deep-seated, serious purulent infections, including brain and liver abscesses (1, 2). This pathogen secretes a human-specific cytolyisin, intermedilysin (ILY), which was originally identified in studies using an *S. intermedius* strain, UNS46, isolated from a human liver abscess (7). ILY is a member of the cholesterol-dependent cytolyisin (CDC) family and is considered the major virulence factor for infectivity and cytotoxicity toward human cells by *S. intermedius* (8–11). 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, two 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 (12). In addition, we recently revealed that *ily* expression and the growth rate of the bacteria are modulated through catabolite control protein A (CcpA), which is a LacI/GalR-type re-

pressor that monitors the extracellular glucose/utilizable carbohydrate concentration (13).

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 (14–17). 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 (17). 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

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TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description, relevant characteristics, or purpose	Reference or source
<i>S. intermedius</i> strains		
PC574	Low-ILY-producing strain from human dental plaque	9
UNS38	High-ILY-producing strain from human brain abscess	11
UNS38 B3	<i>ily</i> knockout strain derived from strain UNS38	11
PC574 Δ <i>lacR</i>	<i>lacR</i> knockout strain derived from strain PC574	This study
<i>E. coli</i> strains		
DH5 α Z1	F [−] ϕ 80dlacZ Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K [−] m _K ⁺) <i>phoA supE44</i> λ [−] <i>thi-1 gyrA96 relA1 tetR lacI^q Spec^r</i>	23
TG1 (der)	F' <i>traD36 lacI^q Δ(lacZ)M15 proA⁺ B⁺ recA::tet supE thi Δ(lac-proAB) km-repA</i>	21
Plasmids		
pGh9:ISS1	Generate random insertions into the chromosome	20
pSETN1	<i>Streptococcus-E. coli</i> shuttle vector	13
<i>placR</i>	pSETN1 carrying <i>lacR</i> isolated from strain NCDO2227	This study
<i>placR</i> (C135Y)	pSETN1 carrying <i>lacR</i> containing a cysteine 135-to-tyrosine mutation isolated from strain P22	This study
<i>placR</i> (S117N, C135Y)	pSETN1 carrying <i>lacR</i> containing a serine 117-to-asparagine and cysteine 135-to-tyrosine mutation isolated from strain HW7	This study
<i>placR</i> (R37L)	pSETN1 carrying <i>lacR</i> containing an arginine 37-to-leucine mutation isolated from strain A4676a	This study
<i>placR</i> (L48F)	pSETN1 carrying <i>lacR</i> containing a leucine 48-to-phenylalanine mutation isolated from strain UNS46	This study
<i>placR</i> (V21D)	pSETN1 carrying <i>lacR</i> containing a valine 21-to-aspartic acid mutation isolated from strain UNS38	This study
<i>placR</i> (R50W)	pSETN1 carrying <i>lacR</i> containing an arginine 50-to-tryptophan mutation isolated from strain UNS35	This study
<i>placR</i> (S117I)	pSETN1 carrying <i>lacR</i> containing a serine 117-to-isoleucine mutation isolated from strain UNS32	This study
<i>placR</i> (V30A)	pSETN1 carrying <i>lacR</i> containing a valine 30-to-alanine mutation isolated from strain UNS45	This study
<i>placR</i> (42Q_44Ldup, C135Y)	pSETN1 carrying <i>lacR</i> containing duplication of a glutamine 42-to-leucine 44 and cysteine 135-to-tyrosine mutation isolated from strain JICC 33616	This study

pathways. Lactose is first internalized by the phosphoenolpyruvate (PEP)-dependent lactose phosphotransferase (PTS) system (lactose-PTS permease, LacFE), yielding lactose-6-phosphate (Lac-6-P). Lac-6-P is then hydrolyzed to glucose and Gal-6-P by a cytoplasmic phospho- β -galactosidase (LacG). Galactose is internalized by the glucose- and lactose-PTS permeases, yielding Gal-6-P. The Gal-6-P generated from these sugars can then be catabolized to glyceraldehyde-3-phosphate and D-glyceraldehyde-3-phosphate by enzymes in the tagatose-6-phosphate pathway (LacA to LacD). It has been reported that these enzymes are encoded by the *lac* operon in some Gram-positive cocci (14–17). The lactose phosphotransferase system repressor (LacR) is a member of the GntR family of transcriptional regulators (18). It has been shown that LacR can repress transcription of the *lac* operon by binding the LacR recognition element, which consists of direct repeats of the sequence TGTNTNWTNT (where N is any base and W is A or T) on the *lac* promoter under lactose- or galactose-limited conditions (18, 19). It is believed that tagatose-6-phosphate, 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 under conditions where lactose or galactose is abundant (17, 18).

AI-2 and CcpA have been reported to regulate *ily* expression. 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 strain from human dental plaque.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. *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 (13). 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 MOPS buffer (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. *Escherichia 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 μ g/ml for *E. coli*; chloramphenicol (Cm), 20 μ g/ml for *E. coli* and 2 μ g/ml for *S. intermedius*; and erythromycin (Em), 100 μ g/ml for *E. coli* and 1 μ g/ml for *S. intermedius*.

Random gene disruption of low-ILY-producing strain PC574. pGh9:ISS1 (Table 1) 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 ISS1 1 to 3), which could generate larger beta-hemolysis zones than strain PC574 on human erythrocyte agar, were used for plasmid rescue experiments.

Plasmid rescue method. Sequences flanking the pGh9:ISS1 insertion site were obtained using a sequence rescue strategy, as described previously (21). Briefly, the chromosomal DNA of *S. intermedius* PC574 ISS1 1 was purified and digested with EcoRI. The digested DNA was self-ligated and

TABLE 2 *S. intermedius* strains used for sequencing of *lacR* and measurement of relative hemolytic activity compared with strain UNS38

Strain ^a	Isolation source(s)	Mutation(s) in <i>LacR</i>	Relative hemolytic activity ^b (%)	Reference or source
A4676a	Brain abscess	R37L	329.9 ± 13.3	9
UNS46	Liver abscess	L48F	187.0 ± 18.2	7
JICC 33405	Empyema, mediastinitis	C135Y	113.2 ± 3.3	This study
UNS38	Brain abscess	V21D	100	9
UNS35	Brain abscess	R50W	91.9 ± 0.9	9
UNS40	Liver abscess	— ^c	82.4 ± 26.5	9
NMH2	Brain abscess	V21D	61.3 ± 2.3	9
UNS32	Liver abscess	S117I	54.5 ± 3.7	9
JICC 1063	Liver abscess	V30A, C135Y	53.0 ± 7.5	This study
UNS45	Liver abscess	V30A	46.1 ± 8.6	9
JICC 40138-2	Infective endocarditis	42Q_44Ldup, C135Y	42.0 ± 2.7	This study
F600	Abdominal abscess	—	48.2 ± 0.5	9
JICC 33616	Brain abscess	42Q_44Ldup, C135Y	34.6 ± 5.8	This study
JICC 32157	Empyema, mediastinitis	C135Y	28.2 ± 0.6	This study
UNS42	Liver abscess	—	27.6 ± 2.2	9
HW13	Abdominal umbilical	—	22.3 ± 0.5	9
UNS27s	Liver abscess	—	15.3 ± 0.9	9
JICC 674	Septicemia (not infective endocarditis)	—	14.4 ± 0.3	This study
HW58	Brain abscess	C135Y	12.9 ± 0.2	9
JICC 32100	Septicemia (not infective endocarditis)	—	12.6 ± 0.7	This study
P58	Gingivitis	—	11.2 ± 0.8	This study
JICC 33404	Pelvic abscess	—	10.6 ± 0.3	This study
JICC 32138	Mediastinitis	—	10.4 ± 0.6	This study
JICC 32132	Brain abscess	—	10.2 ± 0.3	This study
JICC 32122	Brain abscess	—	9.9 ± 0.8	This study
P68	Gingivitis	—	9.4 ± 0.7	This study
CDC415/87	Brain abscess	—	7.8 ± 0.4	9
JICC 33620	Brain abscess	—	7.8 ± 0.2	This study
P22	Gingivitis	C135Y	7.3 ± 0.3	This study
JICC33412	Subcutaneous abscess	—	6.6 ± 0.2	This study
GN472	Dental plaque	—	6.3 ± 0.7	9
HW7	Brain abscess	S117N C135Y	6.3 ± 0.3	9
JICC 689	Infective endocarditis	—	6.3 ± 1.8	This study
JICC 32151	Empyema, mediastinitis	—	5.0 ± 0.3	This study
NMH8	Unknown (wound swab)	C135Y	3.6 ± 0.8	9
E691	Eye	—	3.4 ± 0.1	9
DP101	Dental abscess	—	3.2 ± 0.3	9
HW69	Brain abscess	C135Y	3.2 ± 0.3	9
JICC 32135	Empyema, mediastinitis	C135Y	2.8 ± 0.6	This study
JICC 33425	Subcutaneous abscess	—	2.7 ± 2.4	This study
F458s	Abdominal mass	—	2.4 ± 1.0	9
P101	Gingivitis	—	2.3 ± 0.4	This study
WS100s	Bite wound, hand	—	2.3 ± 0.3	9
JICC 33494	Brain abscess	—	2.2 ± 0.5	This study
PC574	Dental plaque	—	1.8 ± 1.1	9
AC800	Dental plaque	C135Y	1.6 ± 1.1	9
JICC 53299	Suppurative arthritis	—	0.6 ± 0.4	This study
AC5803	Dental plaque	—	0.5 ± 0.1	9
P88	Gingivitis	—	0.3 ± 0.3	This study
F44R	Arm abscess	—	0.1 ± 0.1	9
PC7466	Dental plaque	—	<0.1	9
2Q	Brain abscess	—	<0.1	9
HARDY-DAVID T1	Acute sinusitis	—	<0.1	9
DP102	Dental plaque	—	<0.1	This study
AC4720	Dental plaque	—	<0.1	9
P16	Gingivitis	—	<0.1	This study
NCDO2227	Unknown (type strain)	—	<0.1	9
JICC 253	Septicemia (not infective endocarditis)	—	<0.1	This study

^a High-ILY-producing strains are indicated by boldface type.^b Relative hemolytic activity (see Materials and Methods) showed ILY hemolytic activity in the culture supernatant of strain UNS38 set at 1. Values are means ± standard deviations from 3 replicates each.^c —, no amino acid substitution was observed in the amino acid sequence of *LacR*.

TABLE 3 Oligonucleotides used in this study

Purpose	Oligonucleotide	Sequence (5'–3') ^a
Disruption of <i>lacR</i>	lacR F	GAGGCGTTGAACGTGATACATTTTCGAC
	lacR BamHI R	TGCGGATCCAGTTCTTGAAGAATAACTC
	lacR SalI F	AATGTCGACCGTATACGCGTGTGTTATAG
	lacR R	GATTTTCATCGTACTCATTACCCAATC
	erm BamHI F	AATGGATCCCCGATAGCTTCCGCTATTG
	erm SalI R	CAGTAGTCGACCTAATAATTTATCTAC
Complementation of Δ <i>lacR</i> mutant and 6His-tagged <i>lacR</i>	lacR EcoRI F	CAAGAATTCGGCGTAAAGCTCCACGTTGG
	lacR BamHI F	GAGGATCCATGAAAGAAGGACGACATAGAG
	lacR PstI R	AAAATCACCTGCAGCTTCACGAACAGGTG
Determination of nucleotide sequences of <i>lacR</i>	lacR seq. F	CTTGTTTTGTGTGTCATTTCCAGACTCC
	lacR seq. R	CAGGCTCAATCTAACATAGATGAGACCTG
	lacR seq. F1	GGAATCTAATTATATGATTAGAAAGGAG
	lacR seq. R2	GTCAATCTTTCTTCAAAAAATCACCTGC
Biotinylated DNA probe pulldown assay	Bio-Pily F	Bio-TAGCCGCTTTATCCATCTAACTCTTATCCC
	Pily R	AAATTAGCCTCCTTTTGCTAAATTGCTAAC
	Bio-PlacD F	Bio-TTTGTCTCCTTTCTAATCATATAATTAG
	PlacD R	TCCCAGACTCCTTTTATTTTATATGATTTT
	Bio-PlacA F	Bio-ATCCTCTCCTTCTGTTTATTTGTGTG
	PlacA R	TGTTATACCTCCTTTTCTTTTACAACAAC
	Bio-lacF F	Bio-GAATAGGGAAGAAACAACATTACTTGG
	lacF R	CAGTAAATCAGTCTGTGCACGATGCGCGTC

^a Bio in the sequence stands for biotinylated.

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 primers pGh⁺9#02 and 5'ISS1(rev) (rev stands for reverse) (21). 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.

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.

Generation of *lacR* knockout mutant in strain PC574. A *lacR* knockout mutant (Δ *lacR* mutant) was produced by homologous recombination. 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 3) 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 3) and then digested with SalI. The Em resistance cassette was amplified from the genomic DNA of *ily* knockout mutant UNS38 B3 (11) using primers erm BamHI F and erm SalI R (Table 3). The BamHI- and SalI-digested erythromycin 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 3). 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 μ g/ml Em. Disruption of *lacR* was confirmed by PCR, as well as by immunoblotting using anti-LacR rabbit antiserum (Fig. 1B).

Complementation of *S. intermedius* PC574 Δ *lacR* mutant. *Streptococcus-E. coli* shuttle vector pSETN1 (13, 22) 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 3) 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 (Table 2). The amplified fragments were digested with EcoRI and PstI, cloned into the corresponding sites in pSETN1, and transformed into *E. coli* DH5 α Z1 (Table 1). Each resultant plasmid (Table 1) was transformed into a CSP-treated PC574 Δ *lacR* mutant. Transformants were selected and isolated on BHI agar containing 2 μ g/ml Cm and then

FIG 1 (A) Schematic illustration of the strategy for producing the Δ *lacR* mutant by allelic exchange mutagenesis. *ermF-ermAM* are the Em-resistant genes for an Em cassette. (B) LacR immunoblotting analysis for confirmation of the disruption of *lacR* and its plasmid complementation. *S. intermedius* PC574, PC574 Δ *lacR* mutant containing control vector pSETN1, and PC574 Δ *lacR* mutant transformed with *placR* were cultured in BHI medium for 24 h. Whole-cell extracts (10 μ g) were separated by SDS-PAGE. Immunodetection was carried out with anti-LacR rabbit serum. Lane 1, *S. intermedius* PC574(pSETN1); lane 2, PC574 Δ *lacR*(pSETN1); lane 3, PC574 Δ *lacR*(*placR*).

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 $\Delta lacR$ mutant.

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 ($5,000 \times g$). Isolation of total RNA from cells and quantitative RT-PCR (qRT-PCR) analysis was performed as previously described (13). 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 (13) was used for quantification of *ily* mRNA. The primer set of qRT-*gyrB* F and qRT-*gyrB* R (13) 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 $\Delta lacR$ 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.

Nucleotide sequences of *lacR* from *S. intermedius* clinical isolates. *lacR* fragments containing the putative native promoter were amplified by PCR and sequenced using either primer set lacR seq. F and lacR seq. R or primer set lacR seq. F1 and lacR seq. R2. DNA sequencing was performed by an industrial sequence commission (Hokkaido System Science, Sapporo, Japan).

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 (11, 24). HepG2 cells in 350 μ l 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% CO₂. For cell infection, bacterial cultures were centrifuged at $13,000 \times g$ for 1 min, and the cells were resuspended at a density of 1×10^6 cells in 350 μ l 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 μ l of fresh medium containing 5% FBS and 0.1% human plasma. A portion of the culture medium (200 μ l) 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 (25). After infection, the medium was removed at the indicated time point, and the cells were incubated with 350 μ l of NR solution (50 μ g/ml) in DMEM for 3 h at 37°C. The cells were subsequently washed three times with PBS and then fixed with 200 μ l formaldehyde solution (1.0%, vol/vol) containing 1 mM HEPES-KOH (pH 7.3), 0.85% NaCl, and 1.0% CaCl₂. 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) = $[(A_{540} \text{ of the extract from infected cells} - A_{540} \text{ of the extract from the control for 0\% viability}) / (A_{540} \text{ of the extract from the control with 100\% viability} - A_{540} \text{ of the extract from the control for 0\% viability})] \times 100$.

Human erythrocyte agar plating. Hemolysis induced by the bacterial cells was examined on human erythrocyte agar incubated at 37°C for 1 day under anaerobic conditions. Human blood samples were obtained from healthy Japanese volunteers and stored in an equal volume of sterilized Alsever's solution at 4°C. Before use, the human blood cells (5 ml) in Alsever's solution (5 ml) were washed three times with phosphate-buff-

ered saline (PBS), centrifuged ($1,000 \times g$), and resuspended in 5 ml of PBS. Human erythrocytes suspended in PBS were added to BHI medium containing 1% (wt/vol) agar at a final concentration of 10% (vol/vol).

Hemolysis assay. *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 ($5,000 \times g$) and standardized by dilution with PBS for an optical density at 600 nm (OD_{600}) of 0.25 to 0.5 for the assay. Hemolysis was assayed as previously described (7) with minor modifications. Human erythrocytes stored in sterilized Alsever's solution were washed three times with PBS at 4°C by centrifugation ($1,000 \times g$) before use. Chilled PBS containing 5×10^7 erythrocytes/ml and the dilution series (25- 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 ($1,000 \times g$) at 4°C for 5 min. The A_{540} of 200 μ l 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 = $[(A_{540} \text{ of the supernatant from the sample containing diluted culture supernatant} - A_{540} \text{ of the supernatant from the sample containing no diluted culture supernatant}) / (A_{540} \text{ of the supernatant from the sample completely hemolyzed by hypotonic processing} - A_{540} \text{ of the supernatant from the sample containing no diluted culture supernatant})] \times 100$. 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 $\Delta lacR$ mutant giving 50% of hemolysis) $\times 100$.

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 3). The amplified fragment was digested with BamHI and PstI and cloned into pUHE212-1 (26). The resultant plasmid (pN-his *lacR*) was transformed into *E. coli* DH5 α Z1. Hyperexpression of the recombinant protein was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside to *E. coli* cells in the mid-log phase and by continuing incubation at 37°C for 2 h. The cells were then harvested by centrifugation ($5,000 \times g$) 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 (model XL2020; Misonix Inc., Farmingdale, NY, USA) and then incubated at 30°C for 1 h to denature the proteins. The resultant cell extract was centrifuged at $10,000 \times g$ for 20 min to remove unbroken cells. The supernatant was loaded onto a nickel-nitrilotriacetic 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 500 mM imidazole 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. The renatured and precipitated LacR was frozen at -80°C until use.

Anti-LacR rabbit antiserum. To obtain anti-LacR rabbit antiserum, 150 μ g 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 μ g 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.

Biotinylated DNA probe pulldown assay. Biotinylated DNA probe pulldown assay was performed as previously described with minor modifications (27). Biotinylated DNA fragments were generated by PCR using 5' biotinylated primers (Eurofins MWG Operon, Huntsville, AL, USA) listed in Table 3 and *S. intermedius* NCDO2227 genomic DNA. The *ily* promoter region (213 bp) was amplified using primers Bio-Pily F (Bio stands for biotinylated, and Pily stands for promoter of the *ily* gene) and Pily R. The *lacD* promoter region (168 bp) was amplified using primers

Bio-PlacD F and PlacD R, and the *lacA* promoter region (164 bp) was amplified using primers Bio-PlacA F and PlacA R, respectively. A nonspecific DNA fragment (181 bp) with no LacR recognition element was amplified using primers Bio-lacF F and lacF R. Unincorporated primers were removed using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). A 100- μ l aliquot of a solution of NeutrAvidin (deglycosylated avidin with far less nonspecific binding than biotin) agarose resins (Thermo Scientific, Rockford, IL, USA) was then coated with 1 μ g of biotinylated DNA per the manufacturer's instructions. Whole soluble protein from the cell extract was produced as follows. *S. intermedius* PC574 cells were grown for 16 h in 40 ml of BHI medium. The cells were harvested by centrifugation ($6,000 \times g$, 5 min, 4°C), and the cell pellet was washed twice with 5 ml of lysis buffer containing 10 mM Tris-HCl (pH 7.5) and 50 mM NaCl, and then resuspended in 1 ml of lysis buffer containing protease inhibitor cocktail (EDTA free) (Nacalai Tesque, Tokyo, Japan). The resuspended cells were then disrupted three times using lysing matrix B (Qbiogene Inc., Carlsbad, CA, USA) tubes in a FastPrep cell disruptor (Savant Instruments, Holbrook, NY, USA) at a setting of 6.0 for 20 s each time, with cooling. Debris and undisrupted cells were removed by centrifugation ($14,000 \times g$, 5 min, 4°C), and the total protein concentration of the cleared supernatant was determined using Bradford assay reagent (Bio-Rad, Hercules, CA, USA). The protein concentration was adjusted to 2.0 mg/ml, glycerol was added to a final concentration of 20%, and the solution was stored at -80°C until use.

For the pulldown, an aliquot of DNA-coated resins was mixed with the protein extract (1 mg), and binding buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM KCl, 3 mM MgCl_2 , 20 mM EDTA, 5% glycerol, 40 $\mu\text{g}/\text{ml}$ sonicated salmon sperm DNA, and 10 $\mu\text{g}/\text{ml}$ bovine serum albumin was added to make a total volume of 1 ml. Following a 30-min incubation at room temperature with gentle mixing, the resin was collected by centrifugation ($500 \times g$, 1 min, 4°C), washed four times with 500 μl of binding buffer, and then suspended in 50 μl of sodium dodecyl sulfate (SDS) sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A similar method was used for the pulldown assay using the His-tagged recombinant LacR (5 μg) instead of the whole soluble protein, except for the addition of 0.2% Nonidet P-40 in binding buffer to reduce nonspecific binding of recombinant LacR. LacR precipitated by the resins was visualized by Coomassie brilliant blue staining or by immunoblotting analysis using anti-LacR rabbit serum.

Gel electrophoresis and immunoblotting. *S. intermedius* cells were grown in BHI or MOPS-BHI medium at 37°C under anaerobic conditions. The culture supernatant and cells were separated by centrifugation ($5,000 \times g$). The cells were washed three times with PBS and resuspended in 0.5 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl, 1 mM EDTA, and 10% glycerol. Samples were then added to lysing matrix B (Qbiogene Inc., Carlsbad, CA, USA) tubes and lysed in a FastPrep cell disruptor (Savant Instruments, Holbrook, NY, USA). To obtain the soluble protein fraction, samples were centrifuged at $17,400 \times g$ for 30 min, and the supernatant was retained. Total protein (5 or 10 μg) and LacR, which precipitated along with the biotinylated DNA probe, were subjected to 12.0% SDS-PAGE by the method of Laemmli (28). For immunoblotting analysis, the gel-resolved proteins were transferred to a poly(vinylidene difluoride) membrane (Millipore, Bedford, MA, USA). Blots were incubated with anti-LacR or anti-ILY rabbit serum (9) developed with 5-bromo-4-chloro-3'-indolylphosphate (BCIP)-nitroblue tetrazolium chloride (NBT) by using alkaline phosphatase-conjugated anti-rabbit or anti-mouse immunoglobulin G as the secondary antibody.

Statistics. Data are presented as the mean $\pm$ standard deviation (SD) values.

RESULTS

Identification of a factor that represses *ily* expression. It has been reported that AI-2 and CcpA can regulate expression of *ily*. AI-2 is synthesized by LuxS and is reported to be an exponential-growth-phase-specific activator of *ily*, while CcpA regulates *ily* expression

through carbon catabolite repression (CCR). However, our previous results showed that these control factors could not account for the difference between constitutively high-ILY-producing strains, which seem to be highly pathogenic, and low-ILY-producing strains (data not shown). Therefore, 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:ISS1 with a thermosensitive replicon and transposable element (20). 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*. In addition, we confirmed the localization of plasmid pGh9:ISS1 in the other two high-ILY-producing strains by PCR amplification of *lacR*. The size of the PCR product for these strains was approximately 4,600 bp larger than that expected for the PCR product from the *lacR* gene and corresponded to the presence of pGh9:ISS1. Thus, in all three high-ILY-producing strains, *lacR* was disrupted by pGh9:ISS1 integration, strongly indicating that LacR can repress *ily* expression, i.e., that LacR is a negative regulator of *ily* expression.

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 (Fig. 1A). *S. intermedius* PC574 and *lacR* knockout strain (Δ *lacR* mutant) had a similar colony shape and growth rate in our culture conditions, and hence, a *lacR* mutation has no observable effect on fitness (data not shown). To exclude the possibility that the mutant phenotypes result 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 (*placR*). Immunoblotting analysis using anti-LacR rabbit antiserum was conducted to confirm the deletion of *lacR* and complementation by *placR* (Fig. 1B). The results showed a band corresponding to the molecular mass of LacR (27.7 kDa) from the PC574 cell extract that was not present in the Δ *lacR* mutant cell extract. Recovery of LacR was observed in the cell extract of the Δ *lacR* complementation strain. The level of LacR in the Δ *lacR* complementation strain was virtually that observable in the wild-type cells.

After construction of Δ *lacR* mutant and the complemented strain, the hemolytic activities of these strains were examined on human erythrocyte agar (Fig. 2A). The Δ *lacR* mutant formed a larger beta-hemolysis zone than the wild-type cells. Only a small zone of beta-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. 2B). 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 (Fig. 2B). The higher level of ILY secretion by the Δ *lacR* mutant into the culture medium was also

FIG 2 Hemolysis and *ily* transcriptional activity of $\Delta lacR$ mutant. (A) Hemolytic activity on human erythrocyte agar. *S. intermedium* PC574 and PC574 $\Delta lacR$ mutant transformed with pSETN1 or *placR* (wild type) were inoculated on human erythrocyte agar and then incubated at 37°C for 1 day. (B) Hemolytic activity of the culture supernatant. Cells were grown for 48 h at 37°C in MOPS-BHI medium containing 0.1% glucose. Culture supernatant standardized at an OD₆₀₀ was diluted from 25- to 800-fold by serial 2-fold 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 on the horizontal axis (*x* axis). Symbols: solid circle, *S. intermedium* PC574(pSETN1); open triangle, PC574 $\Delta lacR$ (pSETN1); open square, PC574 $\Delta lacR$ (*placR*). (C) Relative expression levels of *ily*. The strains were grown for 16 h at 37°C in MOPS-BHI medium. The *ily* expression levels in *S. intermedium* PC574(pSETN1) (PC574+pSETN1), PC574 $\Delta lacR$ (pSETN1) ($\Delta lacR$ +pSETN1), and PC574 $\Delta lacR$ (*placR*) ($\Delta lacR$ +*placR*) are indicated relative to the *gyrB* expression level. The results are plotted on a logarithmic scale on the vertical axis (*y* axis). (B and C) The data represent the mean values $\pm$ standard deviations (error bars) of 6 replicates each.

confirmed by immunoblotting using anti-ILY antibody (data not shown).

We also compared the level of *ily* mRNA in *S. intermedium* PC574, $\Delta lacR$ mutant, and the complemented strain by qRT-PCR and measured the relative amounts of *ily* (level of expression of *ily* compared to the level of expression of *gyrB*) in these strains (Fig. 2C). The expression level of *ily* in $\Delta lacR$ mutant cells was 80.7-fold greater than that in PC574 cells and was reduced to a level similar to that in PC574 by *placR* complementation. These results clearly indicate that LacR can repress *ily* expression either directly or indirectly.

LacR binds the *ily* promoter. We performed the biotinylated DNA probe pulldown assay using whole-cell extracts from *S. intermedium* PC574 to determine the possibility of direct interaction between LacR and the *ily* promoter region. In all, four different biotinylated DNA fragments were used for this assay: *Pily*, the *ily* promoter region; *PlacD*, the *lacD* promoter region; *PlacA*, the *lacA* promoter region; and a nonspecific DNA probe. Homology search results showed that both *PlacD* and *PlacA* contain sequences that are very similar to the LacR recognition element in the *lac* operon (Fig. 3A), which differed by only 2 nucleotides compared to the consensus sequence (19). *Pily* and the nonspecific DNA fragment did not show any obvious homology with this element (data not shown). Results from the pulldown assay showed that LacR from the whole-cell extracts coprecipitated with *PlacD* and *PlacA* but did not coprecipitate with the nonspecific DNA probe, as expected (Fig. 3B). Interestingly, LacR coprecipitated with *Pily* at the same efficiency as with *PlacD* and *PlacA*, despite the fact that the *ily* promoter does not have any homology to the LacR recognition element. In addition, similar results were obtained using recombinant LacR (rLacR) which also coprecipitated with *Pily*, *PlacD*, and *PlacA* but not with the nonspecific DNA probe (Fig. 3C). These data strongly suggest that the direct interaction of LacR within *Pily* is a cause of *ily* repression.

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-phosphate, which is a catabolite of lactose and galactose. Since our

data strongly suggest that LacR represses *ily* expression, derepression may occur in the presence of lactose or galactose in the culture medium. To investigate this possibility, PC574 cells were cultured in MOPS-BHI medium supplied with 0.1% glucose, lactose, or galactose (Fig. 4). The amount of ILY secreted into the culture supernatant increased with the addition of lactose or galactose as a carbon source for PC574 cells. The cells cultured with galactose-

FIG 3 (A) Schematic illustration of the *lac* operon. Other components in the tagatose-6-phosphate pathway (*lacC*, *lacE*, *lacF*, and *lacG*) were localized downstream of homologues of phosphotransferase system (PTS) genes. Regions with high homology to the LacR recognition element within the *lac* operon are boxed. *PlacD* is the *lacD* promoter region (168 bp), and *PlacA* is the *lacA* promoter region (164 bp). Nucleotides in regions with high homology to the consensus sequence of LacR recognition element (LacR RE) (N is any base, and W is A or T) are shown in bold capital letters. The predicted $-10/-35$ promoter regions are underlined. (B) Biotinylated DNA probe pulldown assay using the whole-cell extracts from *S. intermedium* PC574. Four different biotinylated DNA fragments were used for this assay: the *ily* promoter region (*Pily*) consisted of the 213-bp region upstream of the *ily* gene translation start site, *PlacD*, *PlacA*, and a nonspecific DNA probe (181 bp). Coprecipitated LacR protein from 5 μ g whole-cell extracts with biotinylated DNA probe was detected by immunoblotting analysis using anti-LacR rabbit serum. Lane 1, nonspecific DNA probe; lane 2, *Pily*; lane 3, *PlacD*; lane 4, *PlacA*; lane 5, LacR exists in 5 μ g whole-cell extracts as the standard marker. (C) Biotinylated DNA probe pulldown assay using the recombinant purified LacR. Five micrograms of recombinant LacR (rLacR) was used for the pulldown assay. Coprecipitated LacR protein with biotinylated DNA probe was detected by Coomassie brilliant blue staining. Lane 1, nonspecific DNA probe; lane 2, *Pily*; lane 3, *PlacD*; lane 4, *PlacA*; lane 5, rLacR (0.2 μ g).

FIG 4 Effects of sugars on ILY secretion. *S. intermedius* PC574 was grown for 48 h at 37°C in MOPS-BHI medium containing 0.1% glucose (solid circle), lactose (open circle), or galactose (open square). Culture supernatant standardized at OD₆₀₀ was diluted from 25- to 1,600-fold by serial 2-fold dilutions, and then the hemolytic activity was measured. The results are plotted on a logarithmic scale on the horizontal axis. Values are means $\pm$ SDs (error bars) for 4 replicates each.

supplemented medium secreted larger amounts of ILY than those cultured with lactose. It is possible that the glucose produced by lactose digestion 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 cells. 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.

Cytotoxicity of Δ lacR mutant on human liver HepG2 cells.

The average level of ILY produced from isolates found in deep-seated abscesses is significantly higher than the levels produced by strains found in normal habitats in contrast to the expression levels of other potential virulence factors such as hyaluronidase and sialidase where no significant difference is observed (9). Moreover, knockout of *ily* or inactivation of ILY in a strain producing high levels of ILY (UNS38) using an anti-ILY antibody showed greatly decreased adherence, invasion, and cytotoxicity of HepG2 cells (11). Therefore, ILY is considered to be the major virulence factor of *S. intermedius*, which is essential for invasion of and cytotoxicity to human cells. It was observed that Δ lacR mutant cells secreted larger amounts of ILY than the wild-type cells did, suggesting that this mutation may result in increased cytotoxicity to human cells. Therefore, we examined the cytotoxicity of the Δ lacR mutant and the complemented strain on the human hepatoma cell line HepG2 (Fig. 5). With the Δ lacR mutant, 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* from *S. intermedius* causes an increase in cytotoxicity, compared to the parental strain, through increased ILY production.

Analysis of the correlation between ILY production and mutation of LacR in clinical isolates. The results shown thus far strongly suggest that hyperproduction and secretion of ILY in the Δ lacR mutant 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

FIG 5 Cytotoxic effect of Δ lacR mutant and its complemented strain on HepG2 cells. Cytotoxic effects were observed over 3 days (d) after bacterial infection. Symbols: solid circle, *S. intermedius* PC574(pSETN1); open triangle, PC574 Δ lacR(pSETN1); open square, PC574 Δ lacR(placR). Values are means $\pm$ SDs for 5 replicates from independent experiments.

NCDO2227 to determine the possible correlations between ILY production and mutations in LacR (Table 2). We classified 13 strains from the 50 strains isolated from clinical specimens as high-ILY-producing strains and determined that these could produce >30% ILY compared to high-ILY-producing strain UNS38 (Table 2). Almost all of the high-ILY-producing strains were from serious, deep-seated abscesses. Among 57 strains, 9 high-ILY-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 2). Two strains had a point mutation at serine 117 of LacR (strains UNS32 and HW7), and 12 strains were mutated at cysteine 135 of LacR. High ILY production was not found in the strains isolated from dental plaques, and only one strain (AC800) had a C135Y mutation in LacR (Table 2). In addition, two high-ILY-producing strains (UNS40 and 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), indicating that an additional factor(s) besides LacR might also play an important role in regulating *ily* expression.

Complementation of Δ lacR mutant by the mutated *lacR*. We further examined whether nine different mutations (R37L, L48F, V21D, 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. 6). Transformation with the mutated *lacR* in the helix-turn-helix domain (R37L, L48F, V21D, 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 12 strains analyzed possessed this mutation (Table 2). 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. However, a high-ILY-producing strain, JICC 33405 produced LacR C135Y at the levels observed with the wild-type strain (data not shown). These data

FIG 6 Complementation of Δ *lacR* mutant by the mutated *lacR*. The *S. intermedius* PC574 Δ *lacR* mutant 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 OD₆₀₀ was diluted from 25- to 800-fold by serial 2-fold dilutions, and the hemolytic activity was measured. Relative hemolytic activity (see Materials and Methods) showed ILY hemolytic activity in the culture supernatant of the *S. intermedius* PC574 Δ *lacR* mutant set at 1. The mutations in plasmid *placR* transforming the *S. intermedius* PC574 Δ *lacR* mutant are shown [e.g., R37L, *placR*(R37L); L48F, *placR*(L48F); V21D, *placR*(V21D); 42Q_44Ldup C135Y, *placR*(42Q_44Ldup, C135Y); S117N C135Y, *placR*(S117N C135Y)]. WT, wild type [PC574 Δ *lacR*(*placR*)]. Values are the means plus SDs (error bars) for 6 replicates from independent experiments.

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. Two LacR mutations of S117I and S117N caused partial reduction in activity; the plasmids expressing these LacR mutations could not suppress the Δ *lacR* phenotype completely. Strain HW7 has S117N and C135Y mutations in LacR; nevertheless, this strain did not show a high-ILY-producing phenotype and secreted only 6.3% ILY compared to strain UNS38 (Table 2). The amount of ILY secreted by strain HW7 was lower than the amount expected after the complementation experiment (Fig. 6). Therefore, this strain may well carry an additional mutation that reduces either the production or secretion of ILY.

DISCUSSION

It had been reported that the genes involved in basic metabolic processes, including the catabolism of complex carbohydrates, are crucial to the pathogenicity of many streptococci (29–32). It is known that CcpA is a major regulator of the expression of carbohydrate catabolism genes and in addition can control the expression of many streptococcal virulence factors (e.g., ILY of *S. intermedius*, streptolysin S, the multiple virulence gene regulator of group A streptococci [GAS], and fructan hydrolase of *Streptococcus mutans*) by CCR (13, 31–35). Therefore, transcriptional control of carbohydrate catabolism genes by CcpA is thought to have an important role in regulating the pathogenicity of streptococci. In this study, we demonstrated by random insertional mutagenesis that another negative transcriptional regulator, LacR, could also control *ily* expression, observed by measuring the enlargement of the zone of hemolysis on human erythrocyte agar as an index. Subsequently, a biotinylated DNA probe pull-down assay

showed that LacR could interact with *Pily* even in the absence of any region of homology with the LacR recognition element (Fig. 3B and C). These unprecedented results suggest that *S. intermedius* LacR might recognize not only this reported consensus sequence but also another unidentified sequence that is localized in *Pily*. Further studies are required to identify this new recognition sequence, which will further our understanding of how LacR controls the expression of *ily* and other genes in *S. intermedius*.

It is well-known that *cdc* genes are found in many Gram-positive pathogens. Nevertheless, to date, CcpA or LacR regulation of *cdc* genes such as *ily* has not been reported, and the mechanisms regulating *ily* expression seem to have evolved specifically in *S. intermedius*. This poses the question as to why this pathogen has evolved this regulation mechanism? The specific binding of ILY to the glycosylphosphatidylinositol-linked membrane protein, human CD59 (huCD59), a regulator of the terminal pathway of complement in humans (36), suggests that *S. intermedius* is primarily adapted to be a human pathogen. It follows then that this bacterium requires horizontal and vertical (mother-to-child) human transmission for successful proliferation within the host population. Our results suggest that *S. intermedius* with a functional LacR has two modes: a less-virulent (low-ILY-producing) mode under conditions when glucose is abundant (13) and a highly virulent (high-ILY-producing) mode under conditions of galactose excess (Fig. 4). In the presence of lactose-abundant foods, such as milk or foods derived from milk, this bacterium might transiently increase its pathogenicity, thereby increasing the chances of successful transmission/colonization as a result of horizontal transmission. Maternal milk contains large amounts of lactose which, in this context might help to promote vertical transmission of this bacterium.

We found that high-ILY-producing strains isolated from severe clinical cases have a substitution of an amino acid(s) and/or an insertion mutation in LacR (Table 2). However, the levels of ILY secreted from 50 clinically isolated strains covered a wide range (Table 2), and 3 high-ILY-producing strains (JICC 33405, UNS40, and F600) had functional LacR (Table 3 and Fig. 6), indicating that *ily* is also regulated by a factor other than LacR. It has been reported that compared to other AGS, infection with *S. intermedius* can cause brain or liver abscesses with high frequency (1, 2). Indeed, 21 of the 50 clinical isolates were derived from these abscesses. We found that 7 strains isolated from liver abscesses secreted elevated levels of ILY, ranging from 15.3% (UNS27s) to 187.0% (UNS46) relative to the high-ILY-producing strain UNS38 (Table 2). Therefore, the increase in ILY production induced by mutation of LacR or some other factor seems to be important for abscess development. However, the levels of ILY secreted from 14 strains derived from brain abscesses were more widely distributed, ranging from <0.1% (2Q) to 329.9% (A4676a) relative to strain UNS38. The processes in the development of a liver abscess by *S. intermedius* (invasion of the human host, survival in neutrophils, and migration into the liver) might require constitutive and higher induction of ILY than that required for the development of brain abscesses. Although, at present, it is unknown whether wild-type strains can benefit from the enhanced production of intermedilysin and resultant increased cell damage by *lacR* mutants, our data showing that the *S. intermedius* PC574 strain and Δ *lacR* mutant have similar growth rates indicate that both could coexist in the same niche. Therefore, in order to further our knowledge of *S. intermedius* pathogenicity, it

is important to investigate possible synergistic partnerships between wild-type and *lacR* mutant strain populations in the human oral cavity (e.g., during tissue invasion). However, as *ILY* is specific to humans, animal models of *S. intermedius* infection are precluded, and alternative strategies, such as the development of human CD59-transgenic mice, will be required in order to study cooperation between these strains.

It has been shown that mutations in the *covRS* (*csrRS*), which encodes a two-component regulatory system, are important in the transition of MIT1 serotype strains from the noninvasive phenotype to the invasive phenotype of *Streptococcus pyogenes* (37). These mutations result in the transcriptional upregulation of multiple virulence-associated genes, including the NAD-glycohydrolase operon for synthesis of the hyaluronic acid capsule and streptolysin O (SLO), streptococcal inhibitor of complement (SIC), and downregulation of the streptococcal pyogenic exotoxin B (SpeB). It was recently reported that 57.3% of *S. pyogenes* strains isolated from group A streptococcal toxic shock syndrome (STSS) contained mutations in *covRS* and/or *rgg* (*ropB*) (38). *Rgg* is also a known repressor of the virulence-associated NAD-glycohydrolase operon, including the gene encoding SLO, and mutation of *rgg* results in the transcriptional upregulation of this operon and downregulation of SpeB, as with *covR* or *covS* mutations (39, 40). SLO is also a member of the CDC family and a known major virulence factor for *S. pyogenes*. Previous studies using a mouse model showed that strains with upregulated SLO induced by *covR* or *covS* mutation could induce necrosis of neutrophils and prompt the escape of mutated strains, resulting in increased lethality (41). Thus, in addition to the upregulation of *ily* expression and *ILY* secretion, mutations in *LacR* could also affect the regulation of other genes/operons associated with virulence of *S. intermedius*. Further studies on the transcriptional control mechanism for *ily* will help us to understand further the mechanisms underlying gene expression and pathogenic phenotype in *S. intermedius*. It had been believed that deep-seated abscesses caused by AGS, including *S. intermedius*, are uncommon in healthy individuals without any identifiable risk factors such as immunocompromised states caused by diabetes, cirrhosis, and cancer (42–44). However, some reports have shown that *S. intermedius* can form deep-seated abscesses in the brain, lung, and spleen in healthy humans (45–47). It is important to analyze whether clinical isolates from such cases show high-*ILY*-producing phenotypes associated with *lacR* mutation.

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