Role of Catabolite Control Protein A in the Regulation of Intermedilysin Production by *Streptococcus intermedius*[▽]

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Streptococcus intermedius **is an opportunistic pathogen of humans that causes purulent infections, including brain and liver abscesses. This pathogen secretes a human-specific cytolysin, intermedilysin, which has been recognized as a major virulence factor. However, most of the expressional control mechanisms of *ily* are still unknown. To determine these mechanisms, we analyzed the nucleotide sequence of the *ily* promoter region. We found a highly homologous region to the catabolite-repressible element (*cre*) in the *ily* promoter region and observed a considerable decrease in the amount of secreted intermedilysin when cells were grown in a culture medium containing high concentrations of glucose/utilizable carbohydrates. Disruption of the *ccpA* gene, which encodes catabolite control protein A, did not induce catabolite repression of *ily* by glucose/utilizable carbohydrates. In *cre* mutants, catabolite repression of *ily* was partially restored, and purified catabolite control protein A bound to an oligonucleotide containing the *cre* consensus sequence in the *ily* promoter region. In addition, a prolonged lag phase and slower doubling time of the *ccpA* mutant cells were observed. Our data show that *S. intermedius* can modulate *ily* expression and growth rate through catabolite control protein A-mediated monitoring of the extracellular glucose/utilizable carbohydrate concentration.**

Streptococcus intermedius is a member of the Anginosus group of streptococci, constituting a part of the normal flora in the human oral cavity and the upper respiratory, gastrointestinal, and female urogenital tracts. The Anginosus group includes opportunistic pathogens that cause purulent infections and abscesses (4, 15, 16, 31, 40, 41). *Streptococcus intermedius* is an important human pathogen and a leading cause of deep-seated infections, including brain and liver abscesses (40, 41). This pathogen secretes a human-specific cytolysin, intermedilysin (ILY), originally identified in investigations using *S. intermedius* strain UNS46 isolated from a human liver abscess (26). ILY is a member of the cholesterol-dependent cytolysins (CDCs), which also include pneumolysin from *Streptococcus pneumoniae*, streptolysin O from *Streptococcus pyogenes*, perfringolysin O from *Clostridium perfringens*, and listeriolysin O from *Listeria monocytogenes* (3, 29). Cytolysins are major virulence factors of the bacteria producing them, and by using toxin gene-deficient or knockout mutants these have been determined to be key factors in infection events (5, 8, 14, 35, 36). ILY is considered 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 (27); (ii) an *ily* knockout strain showed greatly decreased adherence, invasion, and cytotoxicity of human liver (HepG2) cells, and incubating ILY[+] strain UNS38 with antibody to ILY caused drastic reductions in adherence and invasion of the HepG2 cells (36).

Many bacterial species monitor metabolic enzyme activity in order to control the hierarchical utilization of available sugars, a process known as carbon catabolite repression (CCR). The mechanism of CCR in low-GC-content Gram-positive bacteria has been clarified by studies on *Bacillus subtilis* (7, 9, 11, 38). CCR is controlled by the histidine-containing protein (HPr), phosphoenolpyruvate:sugar phosphotransferase system protein, and catabolite control protein A (CcpA), which is a LacI/GalR-type repressor. 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 in turn 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*). Several *in vivo* and *in vitro* studies have shown that the *cre* consensus sequence is WTGNAANCGNWNNCW (N is any base and W is A or T), where the underlined bases are involved in CcpA binding (18, 25, 32, 39). If the *cre* site is located within the promoter region or open reading frame, CcpA binding inhibits

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TABLE 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant characteristic(s)	Reference or source
S. intermedius strains		
UNS38	ILY high-producing strain from human brain abscess	36
UNS38 B3	*ily* knockout strain derived from UNS38	36
UNS38 Δ*ccpA*	*ccpA* knockout strain derived from strain UNS38	Present study
UNS38 EM^r	Insertion of EM cassette in front of *ily* promoter region of UNS38	Present study
UNS38 *creE*	Same as UNS38 but with three point mutations in *cre* site	Present study
UNS38 *creS*	Same as UNS38 but with two nucleotide deletions and one point mutation in *cre* site	Present study
E. coli strains		
C600	*thr-1 leuB6 thi-1 lacY supE44 rfbD1 fhuA21*	Lab collection
DH5αZ1	F⁻ *endA1 hsdR17*(r$_K$⁻ m$_K$⁺) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ(*lacZYA-argF*)*U169 deoR* ϕ80d*lacZ*ΔM15	23
Plasmids		
pSET1	*S. suis* replicating plasmid with Cm resistance	37
pSET1 Δ*lac*	Deletion of *lac* promoter in pSET1	Present study
pSET1 Δ*lac* p15A	pSET1 Δ*lac* carrying p15A replication origin	Present study
p*ccpA*	pSET1 Δ*lac* p15A carrying *ccpA* and putative native promoter	Present study
pUHE212-1	IPTG-inducible expression vector with N-terminal six-His tag	10
pN-his *ccpA*	pUHE212-1 carrying *ccpA*	Present study

the RNA polymerase interaction with the promoter or its progression through to DNA, thereby repressing transcription (19). The regulation of virulence genes by CcpA has been highlighted, and many streptococcal virulence factors (e.g., those for streptolysin S and the multiple virulence gene regulator of group A streptococci [GAS] and fructan hydrolase of *Streptococcus mutans*) have been found to be controlled by CCR (1, 2, 17, 20, 33, 34).

Although only Autoinducer-2 (a LuxS product used by several bacteria in quorum-sensing signaling) has so far been reported to be an activation factor for *ily* expression (30), most of the expressional control mechanisms of *ily* remain unknown. To reveal these mechanisms, we analyzed the nucleotide sequence of the *ily* promoter region for putative control elements. Here, we show that CcpA can control *ily* expression by direct interaction with the *cre* site located 3 nucleotides behind the Pribnow box.

MATERIALS AND METHODS

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 (CO₂:H₂:N₂, 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. An enzyme immunoassay (EIA) and quantitative reverse transcription-PCR (qRT-PCR) were performed using the modified MOPS-BHI medium in which the amount of BHI broth was reduced to 50% of the amount present in the MOPS-BHI medium. *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 at 20 μg/ml for *E. coli* and 2 μg/ml for *S. intermedius*, and erythromycin at 1 μg/ml for *S. intermedius*.

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 (National Institutes of Health). A multiple sequence alignment

was constructed with the Parallel PRRN program (Kyoto University Bioinformatics Center, Japan) (12).

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 was amplified from *ily* knockout mutant UNS38 B3 (36) genomic DNA using primers erm (BamHI) F and erm (SalI) R (Table 2). The BamHI- and SalI-digested erythromycin 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 erythromycin. 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. 5A), an erythromycin 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 SalI-digested erythromycin cassette was ligated with SalI-digested *ily* UP and BglII-digested 3′ *ily* no. 3. The ligated fragment was then amplified by using primers nested PCR F and nested PCR R (Table 2). UNS38 erythromycin 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 erythromycin. Insertion of the erythromycin cassette was confirmed by PCR and sequencing (data not shown).

Mutations were introduced by PCR in the *cre* putative site (Fig. 5B). 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 erythromycin cassette was amplified from UNS38 EM^r genomic
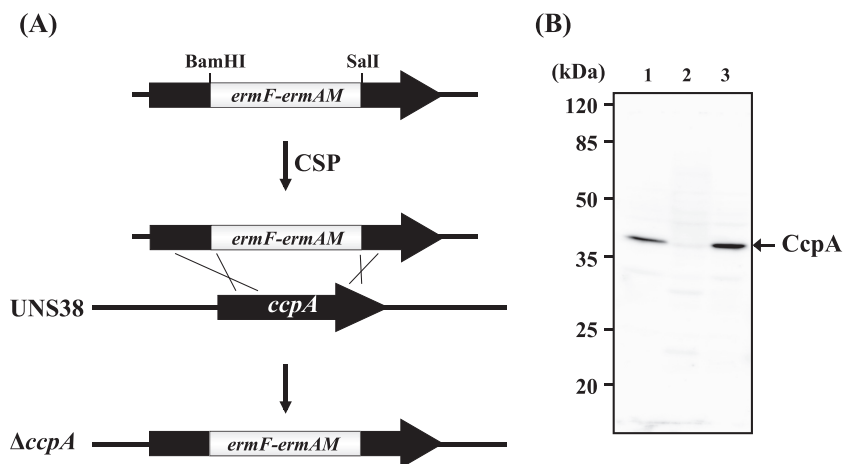
(A)

(B)

FIG. 1. (A) Schematic of the strategy for producing the ΔccpA strain by allelic exchange mutagenesis; *ermF-ermAM*, erythromycin resistance genes for the erythromycin cassette. (B) CcpA immunoblotting analysis for confirmation of the ΔccpA and ΔccpA/p*ccpA* strain. UNS38, ΔccpA, and ΔccpA/p*ccpA* cells were cultured in BHI medium. 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, ΔccpA; lane 3, ΔccpA/p*ccpA*.

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

TABLE 2. Oligonucleotides used in the 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	AAGAATTCAAATGCTTGAAAGTGTTTCCAATAAGTG
	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	cre sense	ATAAATGAAAGCGTTAGCAATTTAGCAAA
	cre antisense	TTTGCTAAATTGCTAACGCTTTCATTTAT
	cre EcoRI sense	ATAAATGAAAGAATTCGCAATTTAGCAAA
	cre EcoRI antisense	TTTGCTAAATTGCGAATTCTTTCATTTAT
	cre SphI sense	ATAAATGAAAGCATGCAATTTAGCAAA
	cre SphI antisense	TTTGCTAAATTGCATGCTTTCATTTAT
	non-cre sense	ATCGGTCTGTTATTTGTGTGTTTTTTATA
	non-cre antisense	TATAAAAAACACACAAATAACAGACCGAT

containing 1 μg/ml erythromycin. Introduction of mutations was confirmed by nucleotide sequencing (data not shown).

Complementation of the *S. intermedius* UNS38 Δ*ccpA* strain. For complementation of the UNS38 Δ*ccpA* mutant, *Streptococcus suis*-*E. coli* shuttle vector pSET1 (37) was modified as follows. 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 *lacZα* 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 by PCR (23), 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 (23). The resultant plasmid (pSET1 Δ*lac* p15A) 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 pSET1 Δ*lac* p15A. The resultant plasmid (pccpA) was transformed into a CSP-treated UNS38 Δ*ccpA* mutant. Transformants were selected and isolated on a BHI agar plate containing 2 μg/ml chloramphenicol. Complementation of Δ*ccpA* was checked by PCR (data not shown) and Western blotting using anti-CcpA mouse antiserum (Fig. 1B).

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).

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 (10). 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 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 XL2020 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 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.

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 (27). 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 (27). Highly reactive monoclonal antibodies were selected and used in ILY immunoblotting and the EIA.

EIA to determine ILY in culture supernatants. *S. intermedius* cells were grown in the modified MOPS-BHI medium containing 0.1% or 1% glucose at 37°C for 24 h under anaerobic conditions, and then the culture supernatant was separated by centrifugation (5,000 × *g*). To estimate the amount of ILY in the culture supernatant of individual strains an EIA was carried out as follows: 50 μl of each culture supernatant diluted with Tris-buffered saline (TBS; 0.9% NaCl and 10 mM TrisHCl [pH 7.4]) was dispensed into the wells of black-masked 96-well EIA plastic plates (ASAHI Glass Co., Ltd., Japan) and dried at 37°C for 24 h. Wells were then blocked with 300 μl/well blocking solution (1% bovine serum albumin in TBS) at room temperature for 1 h. After washing twice with 300 μl/well TBS, 50 μl/well of hybridoma culture supernatant cocktail containing five anti-ILY mouse monoclonal antibodies recognizing different immunodeterminants was dispensed into the wells and reacted with the ILY present at room temperature for 1 h. Each well was washed five times with 300 μl/well TBS. Subsequently, 50 μl/well of 2,500-fold-diluted alkaline phosphatase-labeled anti-mouse IgG(H+L) goat IgG (Promega, Madison, WI) with the blocking solution was dispensed into each well and reacted with any immunocomplex present on the wells at room temperature for 1 h. Each well was then washed five times with 300 μl/well of TBS and 50 μl/well of substrate solution containing 100 mM NaCl, 5 mM MgCl$_2$, and 2 mM 4-methylumbelliferyl phosphate disodium salt (Sigma Chemical Co., St. Louis, MO), and 100 mM Tris-HCl (pH 9.5) was dispensed into each well. After 60 min at 25°C, the fluorescence in each well was measured using an EIA plate reader (TECAN Infinite M200; excitation at 380 nm and emission at 450 nm). Purified ILY prepared as described previously (26) was used to construct a standard curve.

Quantitative RT-PCR analysis. *S. intermedius* cells were grown in the modified MOPS-BHI medium at 37°C for 14 h or 24 h under anaerobic conditions, and then cells were separated by centrifugation (5,000 × *g*). Total RNA was isolated from cells using the FastRNA Pro Blue kit (Qbiogene Inc., Carlsbad, CA) and a FastPrep cell disruptor (Savant Instruments, Holbrook, NY) according to the manufacturer's recommendations. Contaminated chromosomal DNA in the RNA sample was degraded using an RNase-free DNase set (Qiagen GmbH, Hilden, Germany). Degraded DNA and DNase were removed from the RNA sample by using the RNeasy minikit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. The RNA concentration was measured using a BioSpec minispectrophotometer (Shimadzu, Kyoto, Japan). cDNA was synthesized using high-capacity cDNA reverse transcription kits (Applied Biosystems, Warrington, United Kingdom), and cDNA corresponding to 100 ng of input RNA was used as template in each real-time PCR mixture. Real-time PCR was carried out in 96-well plates on an ABI Prism 7000 apparatus with Power SYBR Green PCR master mix (Applied Biosystems, Warrington, United Kingdom) and the primer set of qRT-*ily* F and qRT-*ily* R (Table 2), which are specific for *ily*. The amount of qRT-PCR product of *gyrB* (DDBJ accession no. AB566421) of strain UNS38, which was amplified with the specific primer set of qRT-*gyrB* F and qRT-*gyrB* R, was used as an internal control to normalize the amount of total RNA in each sample. To prepare calibration curves for the primer set, we used cDNA from UNS38 *creE* cultured under high glucose conditions as the template in a five-step dilution (corresponding to 100, 50, 25, 12.5, and 6.25 ng of input RNA). Thermal cycling conditions were as follows: initial denaturation at 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The amounts of target RNAs were calculated from the calibration curves.

Electrophoretic mobility shift assay. Biotin labeling of the 3′ end of single-strand 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 double-stranded oligonucleotide probe were mixed in a solution containing 2 μM non-labeled 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).

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 ($5,000 \times g$). 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 $17,400 \times g$ 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 (21). For immunoblotting analysis, the gel-resolved 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.

Statistics. Data are presented as means ± standard deviations (SD). Statistical comparisons were made using an unpaired two-tailed Student's *t* test.

RESULTS

Production of Δ*ccpA* and its complementation strain. The nucleotide sequence of the coding region of *ily* and its promoter and the potential regulatory region have been reported for strains UNS38 (GenBank accession no. AB212797) and UNS46 (GenBank accession no. AB029317), isolated from brain and liver abscesses, respectively. To investigate the regulation of *ily* expression, the possible regulatory elements around the *ily* promoter region were searched using an 18-bp *cre* consensus sequence, WWTGNAARCGNWWWCAWW (where W is A or T, N is any base, and R is G or A), with alignment of 22 *cre* genes functional in *B. subtilis* (25). Interestingly, a highly homologous region to the *cre* consensus sequence was found 3 nucleotides behind the Pribnow box in the promoter region of both strains UNS38 and UNS46, with 17 bp of the 18-bp potential *cre* sequence of the *ily* promoter corresponding to the *cre* consensus sequence (see Fig. 5A). This result strongly suggested that CcpA could regulate *ily* expression by CCR. The *ccpA* gene was identified from the whole-genome sequence of *S. intermedius* type strain NCDO2227 (K. Kikuchi et al., unpublished data, and encodes a 36.7-kDa protein. A multiple sequence alignment search revealed that NCDO2227 CcpA shares 53% identity with CcpA from *B. subtilis* subsp. *subtilis* strain 168, whereas the degree of homology to CcpA with *S. pneumoniae* ATCC 700669 is 89%. We introduced a *ccpA* knockout mutation in the UNS38 genome by insertion of an erythromycin cassette (Fig. 1A) in order to investigate whether CCR-controlled *ily* expression was observable in this mutant. 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*). Immunoblotting analysis using anti-CcpA mouse antiserum was conducted to confirm the deletion of *ccpA* and complementation by p*ccpA* (Fig. 1B). The immunoblotting result showed a band corresponding to the molecular weight of CcpA from the UNS38 cell extract that was not present in the Δ*ccpA* cell extract. Recovery of CcpA was observed in the cell extract of the Δ*ccpA* complementation strain. The level of CcpA in the Δ*ccpA* complementation strain cells was much higher than that

in the wild-type cells, possibly resulting from the increased *ccpA* copy number due to plasmid complementation.

Effect of *ccpA* knockout on ILY secretion. When *S. intermedius* was cultured in a medium containing a high glucose concentration ($>0.5\%$), the culture pH fell to <5.0 due to lactate accumulation and was accompanied by a loss of ILY activity and a reduction in the level of ILY in the culture supernatant. Therefore, the MOPS-BHI medium was used, maintaining a neutral pH in the culture medium for at least 24 h under the culture conditions.

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 beta-hemolysis 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 much reduced zone of beta-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). UNS38, Δ*ccpA*, and Δ*ccpA*/p*ccpA* cells were cultured in MOPS-BHI medium containing the concentrations of glucose indicated. 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 other preferred carbohydrates such as maltose and sucrose instead of glucose showed a similar repressive effect on ILY production. However, for Δ*ccpA*, an apparent ILY signal was detected even under high concentrations of glucose or other preferred and utilizable carbohydrates. Addition of a nonpreferred carbohydrate such as glycerol did not repress ILY production in either UNS38 or Δ*ccpA* cells (Fig. 2C). These data strongly suggested that CcpA could repress *ily* transcription by CCR. To confirm this possibility, we examined the level of *ily* mRNA in UNS38, Δ*ccpA*, and Δ*ccpA*/p*ccpA* cells under the low glucose condition (0.1%) and the high glucose condition (1.0%) by using qRT-PCR (Fig. 3). To avoid exhaustion of glucose by overgrowing the cells, we used a modified MOPS-BHI medium, in which the amount of BHI broth was reduced to 50% of the amount present in the MOPS-BHI medium. The relative amounts of *ily* (*ily*/*gyrB*) were measured in UNS38, Δ*ccpA*, and Δ*ccpA*/p*ccpA* cells under the low and high glucose conditions. A striking reduction in *ily* expression to 4% of that observed under the low glucose condition was observed in UNS38 and Δ*ccpA*/p*ccpA* under the high glucose condition. However, the level of *ily* expressed by Δ*ccpA* was the same under both high and low glucose conditions; at 24 h the level of *ily* expressed by Δ*ccpA* was 20-fold greater than that expressed by UNS38 ($P < 0.03$) under the high glucose condition. At the 24-h time point examined these strains were in different growth phases (Fig. 4). Therefore, we also compared the amount of *ily* transcript in log-phase cultures (optical density at 600 nm [OD$_{600}$] of approximately 0.4)
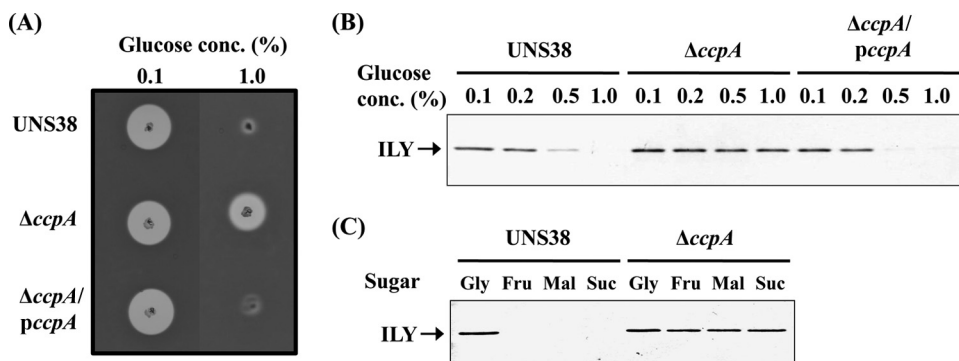
FIG. 2. Effects of glucose/utilizable carbohydrates on ILY secretion by Δ*ccpA* and Δ*ccpA*/p*ccpA* strains. (A) UNS38, Δ*ccpA*, and Δ*ccpA*/p*ccpA* cells were inoculated onto human erythrocyte agar plates containing 0.1% or 1.0% glucose and then incubated at 37°C for 1 day. (B) Cells were grown for 24 h at 37°C in MOPS-BHI medium containing different concentrations of glucose (0.1 to 1.0%). (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 1.0% glycerol (Gly), fructose (Fru), maltose (Mal), or sucrose (Suc), and the OD_{600} of the cultures was measured. Standardized 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.

under the high glucose condition and observed the amount of *ily* mRNA transcribed in Δ*ccpA* to be 4.4-fold higher than that in UNS38 ($P \leq 0.002$) and 9.3-fold higher in Δ*ccpA*/p*ccpA* ($P < 0.001$). We also analyzed the amount of secreted ILY by performing EIA on culture supernatants that were identical to those used for the qRT-PCR experiment (Table 3). A significant amount of ILY was detected in culture supernatants under the low glucose condition in UNS38, Δ*ccpA*, and Δ*ccpA*/p*ccpA* cells, although ILY could not be detected in UNS38 and Δ*ccpA*/p*ccpA* under the high glucose condition. However, Δ*ccpA* secreted the same levels of ILY in the culture medium even under the high glucose condition. These data clearly showed that *ily* expression was regulated by CcpA monitoring of extracellular glucose/utilizable carbohydrate in the culture environment.

During these experiments, we observed that Δ*ccpA* exhibited a slower growth rate than UNS38 and Δ*ccpA*/p*ccpA*. There-

fore, the growth curve of these strains was examined by using BHI medium containing 0.1% or 1.0% glucose (Fig. 4A and B). UNS38 and Δ*ccpA*/p*ccpA* cells had an 8-h lag phase and then grew logarithmically 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 (Fig. 4A and B) had a prolonged lag phase (16 h in 0.1% glucose or 20 h in 1.0% glucose) and then grew logarithmically (until 24 h in 0.1% glucose or 32 h in 1.0% glucose) before entering 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 much longer: 115 min in 0.1% glucose and 85 min in 1.0% glucose.

Isolation and characterization of the *cre* mutants. To confirm that the putative *cre* site serves as a CcpA recognition element (Fig. 5A), two *cre*-mutated strains were constructed to introduce point and deletion mutations into the *cre* site of UNS38 EM[r] (Fig. 5B). 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], UNS38 *creE*, and UNS38 *creS* were inoculated onto human
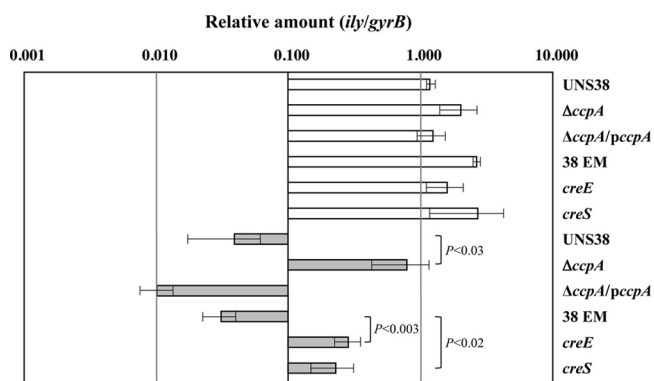

FIG. 3. Relative expression levels of the *ily* gene under low and high glucose conditions. Strains were grown for 24 h at 37°C in modified MOPS-BHI medium with 0.1% or 1.0% glucose. The *ily* expression levels in UNS38, Δ*ccpA*, Δ*ccpA*/p*ccpA*, UNS38 EM[r] (38 EM), UNS38 *creE* (*creE*), and UNS38 *creS* (*creS*) under the low glucose condition (0.1%; open bars) and the high glucose condition (1.0%; gray bars) are indicated relative to the *gyrB* expression level. The results are plotted on a logarithmic scale. The data are plotted as mean values ± standard deviations of three replicates. Statistically significant values ($P < 0.05$) are shown (Student's *t* test).
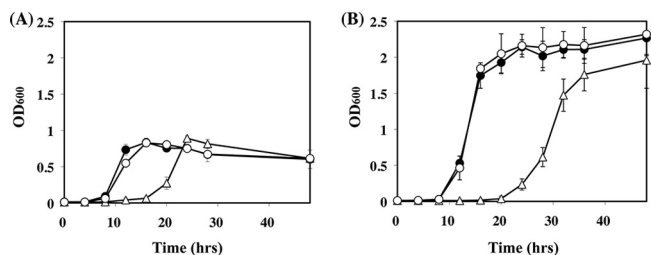

FIG. 4. Growth curves of UNS38, Δ*ccpA*, and Δ*ccpA*/p*ccpA* strains. Strains were cultured in BHI medium with 0.1% (A) or 1.0% (B) glucose, and the OD_{600} was measured at the indicated time points. The graphed data are the mean values ± standard deviations of at least four replicates of independent experiments. Symbols: closed circles, UNS38; open triangles, Δ*ccpA*; open circles, Δ*ccpA*/p*ccpA*.

TABLE 3. ILY secretion into the culture medium

Glucose concn (%)	Amt of ILY secreted[a]					
	UNS38	ΔccpA	ΔccpA/pccpA	38 EM	creE	creS
0.1	8.08 ± 0.04	5.36 ± 0.38	2.50 ± 0.50	4.66 ± 0.46	7.94 ± 0.18	8.90 ± 0.64
1.0	ND	6.44 ± 0.16	ND	ND	1.54 ± 0.10	1.28 ± 0.06

[a] The values shown are means ± standard deviations ($n = 3$) of the amount of ILY (μg/ml/OD$_{600}$ units) in the culture supernatant of individual strains. ND, not detected under the assay condition.

erythrocyte agar plates containing 0.1% or 1.0% glucose. When human erythrocyte agar containing 0.1% glucose was used for the hemolysis assay, the sizes of the surrounding beta-hemolysis 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 beta-hemolysis observed (Fig. 6A). However, a zone of hemolysis was found around the *cre* mutant cells even in the presence of 1.0% glucose.

We further examined the correlation between the amount of ILY secreted and the glucose or other carbohydrate concentration in the culture medium by immunoblotting analysis (Fig. 6B and C). UNS38 EM^r and *cre* mutants were cultured in MOPS-BHI medium containing the indicated concentrations of glucose or other carbohydrates. The amount of ILY secreted into the culture supernatant reduced according to increasing glucose/utilizable carbohydrate concentration 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 glucose/utilizable carbohydrate concentrations, as previously observed for ΔccpA cells. These data strongly suggested that the *cre* site of the *ily* promoter region repressed *ily* expression through the association with CcpA. To confirm this possibility, we examined the relative amount (*ily/gyrB*) of *ily* in UNS38 EM^r, UNS38 *creE*, and UNS38 *creS* cells under the low glucose condition (0.1%) and the high glucose condition (1.0%) by using qRT-PCR (Fig. 3). Identical levels of *ily* expression were observed in UNS38 EM^r, UNS38 *creE*, and UNS38 *creS* under the low glucose condition. In contrast, with UNS38 EM^r under

the high glucose condition a considerable reduction in *ily* expression was observed, to only 1% of that observed with the low glucose level. The levels of *ily* expressed by UNS38 *creE* and UNS38 *creS* were significantly higher (P < 0.02) than those expressed by UNS38 EM^r under the high glucose condition, although these were only 11% (UNS38 *creE*) and 9% (UNS38 *creS*) of the level of *ily* expressed by UNS38 EM^r cultured under the low glucose condition. We also analyzed the amount of secreted ILY by performing EIA on culture supernatants that were identical to those used for the qRT-PCR experiment (Table 3). A significant amount of ILY was detected using EIA from culture supernatants under the low glucose condition in UNS38 EM^r, UNS38 *creE*, and UNS38 *creS*, although ILY could not be detected in UNS38 EM^r under the high glucose condition. UNS38 *creE* and UNS38 *creS* could secrete a detectable amount of ILY in the culture medium even under the high glucose condition, although these amounts were 33% (UNS38 *creE*) and 27% (UNS38 *creS*) of the amount of ILY observed in the culture supernatant of UNS38 EM^r under the low glucose condition. These data showed that the *cre* site of the *ily* promoter region could repress *ily* expression, although *cre* mutants showed only partial derepression of *ily* by CCR. Overall our data suggest that CcpA might control *ily* expression via two pathways: direct repression by binding the *cre* site and indirect repression controlling the amount of transcriptional regulator for *ily*.

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. 7). CcpA requires HPr-(Ser-
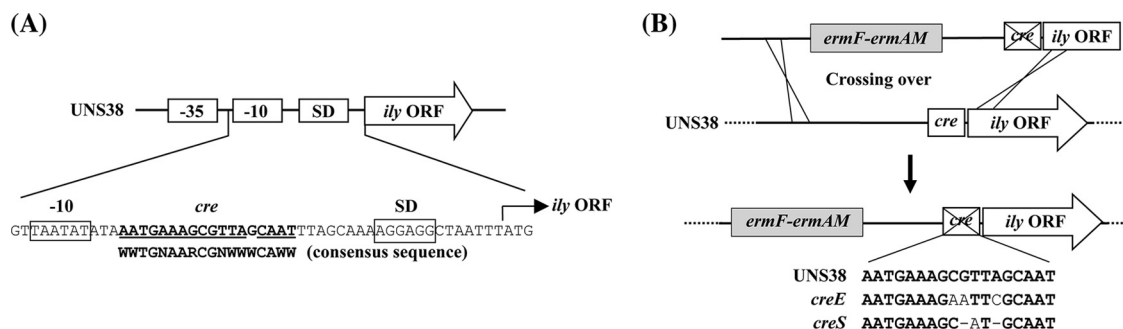

FIG. 5. (A) Schematic of a partial sequence of the UNS38 promoter region. The *cre*-like sequence of the *ily* promoter region and its consensus sequence are shown with bold letters, and the nucleotide sequences corresponding to the consensus sequence are underlined. *ily* ORF, *ily* open reading frame 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) 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).
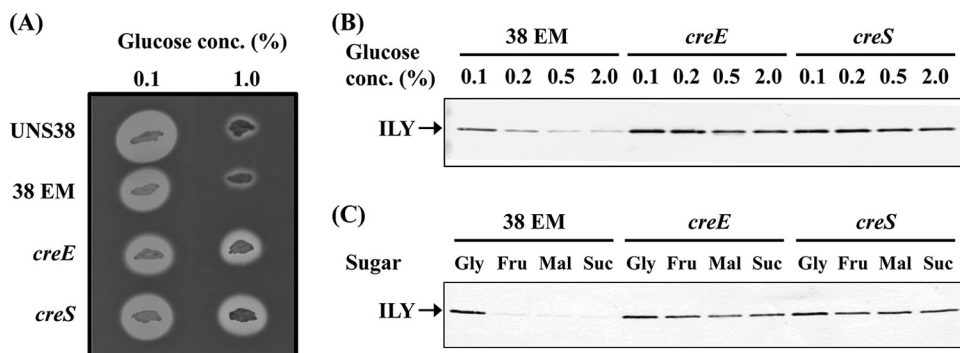
FIG. 6. Effects of glucose/utilizable carbohydrates on ILY secretion by the *cre* mutant strains. (A) UNS38, 38 EM, *creE*, and *creS* cells were inoculated onto human erythrocyte agar plates containing 0.1% or 1.0% glucose and then incubated at 37°C for 1 day. (B) Cells were grown for 20 h at 37°C in MOPS-BHI medium containing different concentrations of glucose (0.1 to 2%). (C) UNS38, 38 EM, *creE*, and *creS* cells were grown for 15 h at 37°C in MOPS-BHI medium containing 0.1% glucose and 1.0% glycerol (Gly), fructose (Fru), maltose (Mal), or sucrose (Suc). Then, the OD$_{600}$ of the cultures was measured, and standardized amounts of the culture supernatants were analyzed by 12% SDS-PAGE. Anti-ILY monoclonal antibody was used as a probe for immunodetection of ILY.

46-P) for binding to the *cre* site *in vivo* (38). Fructose-1,6-bisphosphate is an allosteric effector of CcpA and enhances the binding of CcpA to the fructan hydrolase (*fruA*) gene promoter region carrying intact *cre* of *S. mutans* (1). However, several reports have shown that although there is slightly low affinity, CcpA can recognize and bind to the *cre* without HPr-(Ser-46-P) and fructose-1,6-bisphosphate *in vitro* (1, 2). Therefore, we performed the assays without HPr-(Ser-46-P) and fructose-1,6-bisphosphate. In addition, to avoid nonspecific binding of the biotin-labeled probes to CcpA, we carried out every experiment in the presence of 2 μM nonspecific double-stranded oligonucleotide; this amount was 400-fold higher than that present in the biotin-labeled probes. As shown in Fig. 7, CcpA induced a shift in the mobility of the DNA fragment containing the intact *cre* sequence (Bio-*cre*). However, when fragments carrying a mutated *cre* sequence (Bio-*creE* or Bio-*creS*) were used, a dramatic reduction was observed in the ability of CcpA to induce a shift. These data clearly showed that *ily* expression can be directly controlled by the binding of CcpA to the *cre* site of the *ily* promoter region.
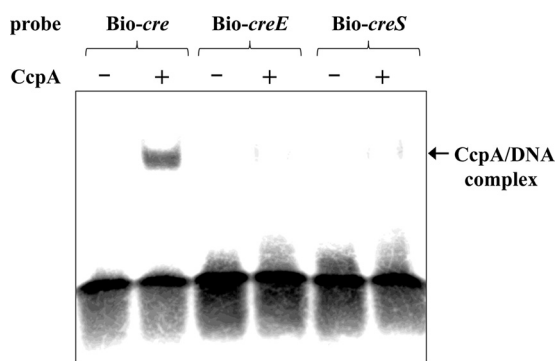

FIG. 7. Electrophoretic mobility shift assay results of biotin-labeled DNA fragments containing wild-type (Bio-*cre*) or mutated (Bio-*creE* or Bio-*creS*) *cre* sequences. Labeled DNA fragments (5 nM) were incubated with (+) or without (-) purified N-his CcpA (100 nM) and then electrophoresed on a 4% nondenaturing acrylamide gel. The labeled DNA was detected by chemiluminescence.

DISCUSSION

ILY is a major virulence factor that is essential for cytotoxicity and invasion of human cells by *S. intermedius*. 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. Therefore, we produced Δ*ccpA* and *cre* mutant strains to investigate whether *ily* expression is controlled by CcpA-dependent CCR. We showed that the Δ*ccpA* mutant did not induce catabolite repression of *ily* by glucose/utilizable carbohydrates, and *cre* mutants could partially restore catabolite repression of *ily*. ILY is a member of the CDCs, and *cdc* genes are found in many Gram-positive pathogens; however, the direct regulation of *cdc* genes by CcpA has so far not been reported. Indeed, the *cre* consensus sequence has not been found up to 500 bp upstream from the translation start site of *cdc* genes such as pneumolysin from *Streptococcus pneumoniae* P1031, suilysin from *Streptococcus suis* P1/7, listeriolysin O from *Listeria monocytogenes* EGD-e, perfringolysin O from *Clostridium perfringens* strain 13, or vaginolysin from *Gardnerella vaginalis* ATCC 14019 (data not shown). Because localization of the *cdc* genes in the genome sequence of the regulatory element and promoter region is generally not conserved even in streptococci, CCR of the *ily* gene seems to have evolved exclusively in *S. intermedius*.

Most of the streptococcal genes regulated by CcpA revealed in the *S. pyogenes* M1 MGAS5005 genome by using transcriptome analysis are similar to the genes reported in a study on *Bacillus subtilis*, many of which are known to be involved or putatively involved in carbohydrate transport and metabolism (20). Carbohydrate catabolism genes mediating transcriptional control by CcpA are thought to have an important function in regulating the pathogenicity of streptococci. 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 (33). In addition, the numerous genes encoding proteins with known or putative carbohydrate metabolism functions are upregu-

lated during GAS and *S. pneumoniae* infections (13, 28). Several recent reports have indicated that CcpA regulates not only the gene for catabolism but also some genes for virulence factors, including streptolysin S of GAS (20, 34). The broad-spectrum cytolysin streptolysin S is a homologue of bacteriocin, which encodes *sagA* and localizes in the *sagA-I* operon of the GAS chromosome (6); *sagA* encodes a 53-amino-acid protoxin and then converts to a 30-amino-acid active cytolysin by modification of the SagBCD trimeric complex and cleavage of the leader sequence (22, 24). Streptolysin S is a major pathogenic factor and is believed to have important functions related to the pathogenesis of streptococcal necrotizing soft tissue infection. Therefore, the fact that both unrelated cytolysins, ILY and streptolysin S, are similarly controlled by CcpA with synchronization of expression of metabolic genes for carbohydrate utilization is suggestive of some important function of ILY in pathogenesis by *S. intermedius*.

BHI medium, a nutrient-rich medium, is commonly used for *S. intermedius* culture. We compared the growth of wild-type strain and Δ*ccpA* in BHI medium containing 0.1% or 1.0% glucose and observed a prolonged lag phase and slower doubling time under both conditions (Fig. 4A and B). A similar but not identical phenotype was observed in Δ*ccpA* from GAS, which grew normally in a nutrient-rich medium; however, a prolonged lag phase was observed in a chemically defined medium with glucose and slower doubling time in a chemically defined medium with maltose (34). These results suggest that CcpA functions to control growth rate by monitoring the extracellular glucose concentration. Addition of 1.0% preferred carbohydrate such as maltose and sucrose into MOPS-BHI medium markedly decreased *S. intermedius* ILY secretion, as observed with glucose; however, such an effect was not observed in Δ*ccpA* (Fig. 2C). In contrast, *cre* mutants showed only partial derepression of *ily* repression by glucose (Fig. 3 and Table 3). CcpA might control *ily* expression via two pathways: direct repression by binding the *cre* site and indirect repression controlling the amount of transcriptional regulator for *ily*. Although these data do not unequivocally demonstrate the influence of CCR/CcpA in real *S. intermedius* infections, they do give grounds to hypothesize that *S. intermedius* has two modes of growth: a rapid growth and lower-virulence mode under preferred carbohydrate-abundant conditions and a slow-growing and highly virulent mode under carbohydrate-limited conditions. It may be that the changing growth phase according to the environment would be a sound strategy for long-term parasitism by using ILY to procure nutrients from the host cells.

In conclusion, we have shown that ILY production is modulated by CcpA-mediated CCR. Further studies elucidating the meaning of simultaneous regulation of carbohydrate catabolism genes and *ily* in *S. intermedius* would help to clarify the strategy for survival, infectivity, and pathogenesis in humans.

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