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Transcriptional Regulation of the *Streptococcus salivarius* 57.I Urease Operon

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The *Streptococcus salivarius* 57.I *ure* cluster was organized as an operon, beginning with *ureI*, followed by *ureABC* (structural genes) and *ureEFGD* (accessory genes). Northern analyses revealed transcripts encompassing structural genes and transcripts containing the entire operon. A σ^{70}-like promoter could be mapped 5′ to *ureI* (*PureI*) by primer extension analysis. The intensity of the signal increased when cells were grown at an acidic pH and was further enhanced by excess carbohydrate. To determine the function(s) of two inverted repeats located 5′ to *PureI*, transcriptional fusions of the full-length promoter region (*PureI*), or a deletion derivative (*PureI*Δ100), and a promoterless chloramphenicol acetyltransferase (CAT) gene were constructed and integrated into the chromosome to generate strains *PureI*CAT and *PureI*Δ100CAT, respectively. CAT specific activities of *PureI*CAT were repressed at pH 7.0 and induced at pH 5.5 and by excess carbohydrate. In *PureI*Δ100CAT, CAT activity was 60-fold higher than in *PureI*CAT at pH 7.0 and pH induction was nearly eliminated, indicating that expression was negatively regulated. Thus, it was concluded that *PureI* was the predominant, regulated promoter and that regulation was governed by a mechanism differing markedly from other known mechanisms for bacterial urease expression.

Ureases are multisubunit enzymes requiring Ni^{2+} for catalytic activity. Several bacterial urease gene clusters have been isolated and characterized, and similarities in the organization of the clusters between species have been demonstrated (17, 18). Most bacterial ureases consist of three subunits, α, β, and γ, encoded by *ureC*, *-B*, and *-A*, respectively (18), although exceptions exist (10). The assembly of a catalytically active urease also requires *ureE*, *-F*, *-G*, and *-D*, known as accessory genes, which encode proteins required for the productive incorporation of Ni^{2+} into the metallocenter within the active site. In some cases, high-affinity nickel transporters, for instance, NixA of *Helicobacter pylori* (16), are known to be required for optimal urease activity. Additional genes, such as *ureI* of *H. pylori* and *ureH* and *ureI* of *Bacillus* sp. strain TB-90, have been identified (15), but the function(s) of these gene products in urease biogenesis is not well defined. However, UreH from *Bacillus* has homology with the high-affinity nickel transporter of *Alcaligenes eutrophus*, HoxN (31); thus, it has been proposed that UreH is involved in energized nickel uptake in conjunction with UreI.

The expression of bacterial urease genes is regulated by different mechanisms (7). Few known bacterial ureases are expressed constitutively, whereas most are regulated by environmental conditions. For example, urease expression in *Klebsiella pneumoniae* and *Klebsiella aerogenes* is activated only under nitrogen-limiting conditions (8, 14, 19), and expression in *Proteus mirabilis* is induced by urea and is mediated by the positive transcriptional regulator UreR (20). Information regarding the expression of ureases of oral bacteria is just beginning to accumulate. Among the species of oral bacteria that have been identified as ureolytic, *Streptococcus salivarius* is believed to be a major contributor to total oral ureolysis (25). In contrast to studies of other known mechanisms for urease expression, previous studies in our laboratory and in others demonstrated that urease expression in *S. salivarius* 57.I is regulated by the environmental pH (4, 27), growth rate, and carbohydrate availability (4). At a neutral pH, expression is almost completely repressed. Induction occurs when cells are grown at an acidic pH, and expression is further enhanced by excess carbohydrate and higher growth rates. Furthermore, based on the results of Northern blot analysis measuring *ureC*-specific mRNAs from cells grown at various pH values and in conditions with limited or excess carbohydrate, it was found that the induction is regulated, at least in part, at the transcriptional level (4).

Our initial attempts to isolate the urease genes from *S. salivarius* 57.I resulted in the cloning of a partial gene cluster that contained the 3′ end of *ureI* followed by *ureABCEFGD* (5). Despite the lack of the 5′ end of the cluster, *Streptococcus gordonii* DL1, a nonureolytic oral microorganism, expressed urease activity when harboring this partial *ure* cluster on a moderate-copy-number plasmid (pMC17) (Fig. 1; Table 1) and when the growth medium was supplemented with $NiCl_2$ (5). The purpose of this work was to identify additional genes that may be involved in urease biogenesis, to investigate the transcriptional organization of the *ure* cluster of *S. salivarius* 57.I, and to explore the basis for differential expression of *ure* genes in response to pH and carbohydrate availability.

Isolation and nucleotide sequence analysis of the 5′ portion of *ureI* and the flanking region from *S. salivarius* 57.I. A 7.8-kbp *Sau*3A fragment containing the 3′ portion of *ureI* and complete *ureABCEFGD* has been previously described (5). No genes involved in urea metabolism were found within 1.5 kbp 3′ to the *ure* cluster, and a putative Rho-independent terminator was identified 120 bases 3′ to the stop codon of *ureD*, the last gene in the cluster. To obtain the complete *ureI* and other genes potentially involved in urease biogenesis, total chromosomal DNA was isolated from *S. salivarius* 57.I and digested to completion with *Xba*I. DNA fragments were separated on aga-

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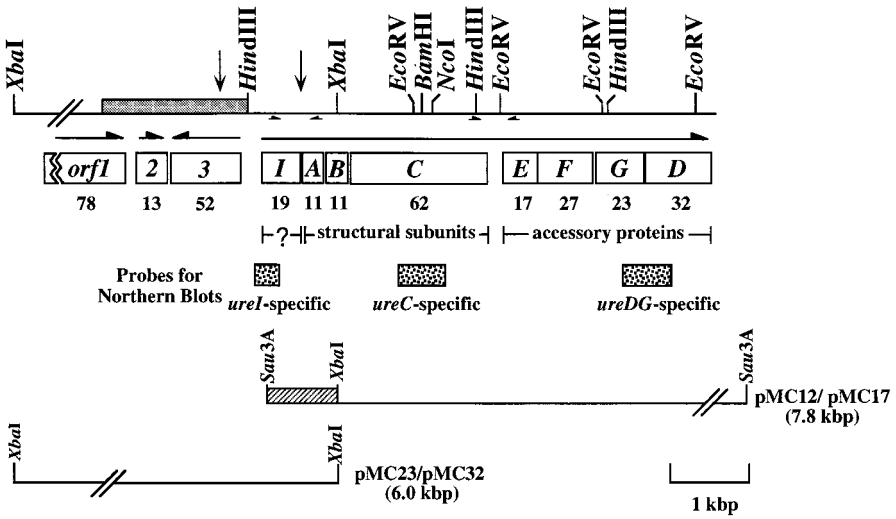

FIG. 1. *ure* cluster and the 5′ flanking region on the *S. salivarius* 57.I chromosome. A restriction endonuclease map of the chromosome containing the *ure* cluster and the 5′ flanking region is shown on the top line. The limits of the DNA sequence shown in Fig. 2 are indicated by vertical arrows. The relative location and the direction of transcription of each ORF are indicated by horizontal arrows. The molecular mass in kilodaltons of each gene product is shown below each gene. The locations and orientations of primer pairs used in RT-PCR are indicated by arrows immediately under the restriction map. The *Sau*3A-*Xba*I region from pMC12, used as a probe for identifying pMC32, is indicated in a hatched box. The region used for the construction of pCW45 and pMC77 (see below) for integration is indicated in a shaded box within the restriction map.

rose gels, transferred to nitrocellulose, and hybridized at high stringency (12) to a radiolabeled *Sau*3A-*Xba*I fragment, approximately 1.0 kbp in size, which contained the 3′ portion of *ureI* (Fig. 1). An approximately 6.0-kbp fragment was subsequently identified (data not shown). To isolate this fragment, a subgenomic DNA library was constructed in pSU21 (2) and screened with the same probe, as described above. The resulting chimeric plasmid was designated pMC23. To maintain stably the *Xba*I fragment in *Escherichia coli*, it was necessary to subclone the insert onto a low-copy-number vector, pDL290

TABLE 1. Bacterial strain and plasmids

Strain or plasmid	Relevant phenotype[a]	Description	Source or reference
S. salivarius			
57.I	Ure^+	Wild-type host	25
*PureI*CAT	CAT^+ Ure^+	Wild-type chromosome containing a single copy of *PureI-cat*	This study
*PureI*Δ100CAT	CAT^+ Ure^+	Wild-type chromosome containing a single copy of *PureI*Δ100-*cat*	This study
S. gordonii			
DL1		Wild-type host	11
MC17	Ure^+ Sp^r	DL1 harboring pMC17	5
E. coli DH10B		General cloning host	LTI
Plasmids			
pGEM-5Zf(+)	Ap^r	General cloning vector	Promega
pGEM-7Zf(+)	Ap^r	General cloning vector	Promega
pUC18	Ap^r	General cloning vector	LTI
pSU21	Cm^r	Moderate-copy-number *E. coli* vector	2
pSF143	Tc^r	Integration vector for streptococcal species	29
pDL278	Sp^r	*Streptococcus-E. coli* shuttle vector	13
pDL290	Km^r	Low-copy-number *E. coli* vector	10a
pCW24	Ap^r Cm^r	pUC18-promoterless *cat*	This study
pCW42	Ap^r Cm^r	pGEM-5Zf(+)-promoterless *cat*	This study
pCW45	Tc^r Cm^r	pSF143-*PureIcat*	This study
pMC12	Cm^r	pSU21-*ureIABCEFGD*[b]	5
pMC17	Sp^r	pDL278-*ureIABCEFGD*[b]	5
pMC23	Cm^r	pSU21-6.0-kbp *Xba*I fragment	This study
pMC32	Cm^r Km^r	pDL290-6.0-kbp *Xba*I fragment	This study
pMC63	Ap^r Cm^r	pCW42-*PureI*	This study
pMC68	Ap^r Cm^r	pCW42-*PureI*Δ100 + 2.0-kbp fragment	This study
pMC71	Ap^r Cm^r	pCW42-*PureI* + 2.0-kbp fragment	This study
pMC77	Ap^r Tc^r	pSF143-*PureI*Δ100-*cat*	This study

[a] Abbreviations: Ure, urease; Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sp, spectinomycin; Tc, tetracycline; r, resistance.
[b] The 5′ end of *ureI* is incomplete.

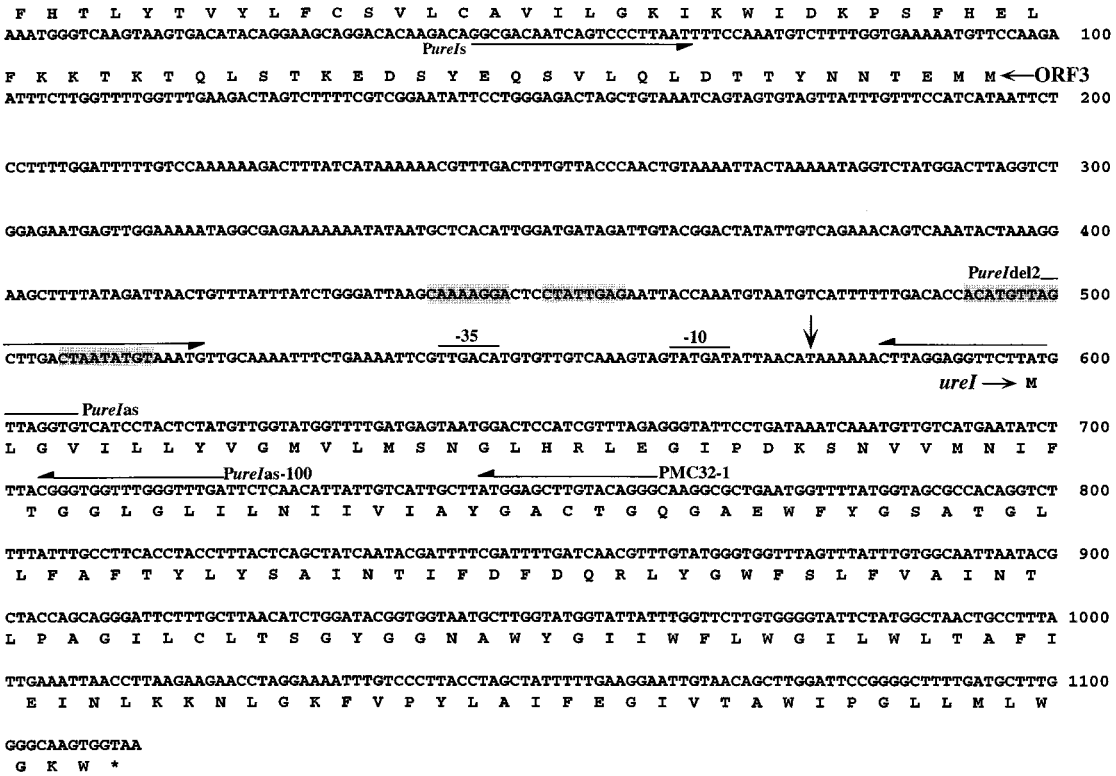

FIG. 2. Nucleotide and deduced amino acid sequences of the 5′ flanking region of the *ure* cluster. ORF3 and *ureI* are transcribed from the opposite DNA strands; thus, the sequence of *ureI* presented here is the coding strand, and the sequence of ORF3 is the noncoding strand. The locations and orientations of primers (P*ure*Ias-100 and PMC32-1) used to identify the transcriptional start site of *ureI* and of primers used to amplify *PureI* and *PureI*Δ100 (P*ure*Is, P*ure*Ide12, and P*ure*Ias) are indicated by horizontal arrows. The transcriptional start site of *ureI*, determined by primer extension analysis, is indicated by a vertical arrow, and the corresponding −10 and −35 regions are overlined. The sequences of the inverted repeats 5′ to *PureI* are shaded.

(10a), which generated pMC32. For convenience, all strains and plasmids used in this study are listed in Table 1.

The complete nucleotide sequences of both strands of the *Xba*I fragment were obtained, and the sequences of *ureI* and the 5′ flanking region are presented here. The *ure* cluster began with an open reading frame (ORF) of 513 bp, which had homology with *H. pylori ureI* and thus was designated *ureI* (Fig. 2). A putative ribosome binding site (RBS) could be found 6 bases 5′ to the predicted start codon of *ureI*. The *ureI* gene was predicted to encode a protein with an estimated molecular weight of 18,995 and a pI of 7.0. In addition, the hydropathy plot of the deduced amino acid sequences indicated that UreI was relatively hydrophobic, with six potential transmembrane domains (data not shown), suggesting that UreI could be a membrane protein.

Translation of the nucleotide sequences of the *Xba*I fragment revealed three additional ORFs located 5′ to the *ure* cluster. ORF1 and -2 were transcribed in the same direction as the *ure* cluster, and ORF3 was transcribed in the opposite direction (Fig. 1). Proposed RBSs were found in the appropriate positions 5′ to all ORFs. ORF1, 2,094 bp in size, encoded a protein with a calculated molecular weight of 78,431 and an estimated pI of 8.9. A high degree of homology was observed between ORF1 and ATP-binding cassette transporters. ORF2, located 109 bp 3′ to ORF1, was 330 bp and encoded a protein with a calculated molecular weight of 12,717 and an estimated pI of 9.6. Based on the hydropathy plot, ORF2 may encode a membrane protein; however, only low levels of similarity were observed between the ORF2 product and other known proteins. ORF3, 1,392 bp in size, was transcribed in the direction opposite to the transcription of ORF1 and -2, and it encoded a protein with a calculated molecular weight of 52,393 and an estimated pI of 9.7. Due to the proximity of these three ORFs to the *ure* cluster and their characteristics, the possibility of their involvement in optimal urease expression, perhaps through Ni^{2+} uptake, is currently under investigation.

Operonic arrangement of the *S. salivarius* 57.I *ure* cluster. To analyze the transcriptional organization of the *ure* cluster and potentially identify a transcript(s) that is induced at a low environmental pH, Northern blot analyses were performed with batch-grown cells at pH 7.0, 6.0, and 5.0, and *ureI*-, *ureC*-, and *ureDG*-specific mRNAs were examined (Fig. 1 and 3A). Total RNA was isolated according to the method of Putzer et al. (21) with modifications. Briefly, cells were cultured in brain heart infusion (BHI; Difco, Detroit, Mich.) containing 50 mM potassium phosphate buffer, pH 7.5, in BHI alone, or in BHI which had been adjusted to pH 5.5 by addition of 2 N HCl to mid-log phase, at which point the cultures were at approximately pH 7, 6, and 5, respectively. Cells were harvested, washed once with 10 mM sodium phosphate buffer, pH 7.0, and then resuspended in 1/40 of the original culture volume in 50 mM Tris–10 mM EDTA, pH 8.0. In 2.0-ml screw-cap microcentrifuge tubes, 0.5 g of glass beads (0.1-mm diameter), 500 μl of concentrated cell suspensions, 500 μl of phenol-chloroform (5:1, pH 4.7; Ambion, Austin, Tex.), and 100 μl of 10% sodium dodecyl sulfate were added. Cells were then subjected to mechanical disruption by homogenization in a Bead Beater (Biospec Products, Inc., Bartlesville, Okla.) for a total of 40 s at 4°C. The aqueous phase was first extracted with an equal volume of phenol-chloroform four to five times, followed

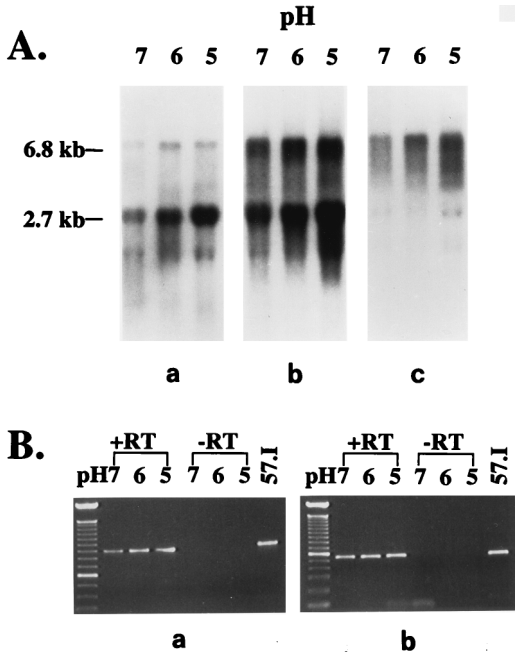

FIG. 3. (A) Northern blot analysis of *ure*-specific messages. Ten micrograms of total cellular RNA from cultures at each pH level was probed with a *ureI*-specific probe (a), a *ureC*-specific probe (b), and a *ureDG*-specific probe (c). (B) PCR products generated from RT-PCR. 1% of total cDNA generated by RT-PCR from each RNA sample was amplified with specific primers (Fig. 1), and 10% of the PCR products were run on a 0.8% Tris-borate-EDTA gel. Some PCR products were generated with a primer pair specific for the *ureIA* intergenic region (a), and others were generated with a primer pair specific for the *ureCE* intergenic region (b). RT was included in some reactions (+RT), but not in control reactions which were carried out identically to the experimental samples (−RT). In other control reactions PCRs were used to amplify the target region from *S. salivarius* 57.I chromosomal DNA under the same conditions (57.I). The 100-bp DNA ladder was used as the molecular weight marker.

by extraction with chloroform-isoamyl alcohol (24:1) alone. Total cellular RNA was then precipitated with 1/10 volume of 3 M Na acetate, pH 6.0, and 2 volumes of ice-cold 99% ethanol. Northern blot analysis was performed with 0.7% formaldehyde-agarose gels (22), and hybridization conditions were as described previously (1).

Two species, approximately 6.8 and 2.7 kb, were identified with a probe specific for *ureI*. The intensity of both signals increased when the culture pH became acidic, and the highest levels were observed in cells grown at pH 5.0. Furthermore, under all growth conditions, the smaller transcript was more abundant than the larger one. Extrapolating from the estimated sizes of RNA transcripts and the known sizes of the urease genes, the two transcripts presumably corresponded to *ureIAB CEFGD* and *ureIABC*, respectively. When total cellular RNA was probed with a *ureC*-specific probe, close examination revealed four fragments, 6.8, 6.3, 2.7, and 2.2 kb, presumably corresponding to *ureIABCEFGD*, *ureABCEFGD*, *ureIABC*, and *ureABC*, respectively. However, due to degradation of the mRNA, which is commonly observed with RNA from oral streptococci, it was not possible to definitely show by Northern analysis that the transcripts could arise from two promoters. The intensities of the signals increased when the growth pH was acidic. However, it was difficult to determine whether all transcripts were enhanced at an acidic pH or only transcripts arising from the promoter 5′ to *ureI* (*PureI*). In agreement with the above results, only the larger two transcripts, 6.8 and 6.3 kb, were observed when a *ureDG*-specific probe was used. Although the signals were not as sharp as those seen in *ureI*- and *ureC*-specific messages, increases in intensity of signals were observed in cells grown in acidic media. We did not observe any transcripts of the appropriate size for *ureEFGD*, suggesting the absence of a functional promoter 5′ to the accessory genes. In addition, all attempts, including the use of primer extension analysis and promoterless reporter fusions, failed to identify functional promoters within the *ureCE* intergenic region.

To confirm that transcription could extend through the *ureIA* and *ureCE* intergenic regions, the presence of the larger transcripts was verified by reverse transcriptase (RT) PCR. cDNA was synthesized from 10 μg of total RNA isolated from cells grown under different conditions with Moloney murine leukemia virus RT and was further amplified by PCR with random hexamers according to standard procedures (9). Negative controls included reactions in the absence of RT. cDNAs spanning the *ureIA* and *ureCE* intergenic regions were detected by PCR with primer pairs with appropriate sequences. In both cases, PCR products with appropriate sizes (Fig. 3B) and correct sequences (data not shown) were obtained, further supporting the operonic arrangement of the *ure* cluster. No products were seen in control reactions that were designed for detecting chromosomal DNA contamination.

It has been proposed that, after activation of the urease apoenzyme, the accessory proteins are released from the holoenzyme and are recycled for the purpose of assembling another catalytically active urease (18). Consequently, the quantity of structural proteins (UreABC) could be greater than that of accessory proteins (UreEFGD) at any given time. Results obtained with Northern analysis are consistent with the theory that transcripts containing the accessory genes are less abundant than those carrying only the structural genes. Although the molecular basis for the generation of the smaller transcripts (*ureIABC* and *ureABC*) is not clear, a strong stem-loop structure ($\Delta G = -34$ kcal/mol) followed by a string of uridine residues was located 11 bases 3′ to the stop codon of *ureC*. We did not observe any transcripts with sizes corresponding to *ureEFGD*; thus, it is not likely that the smaller transcripts resulted from posttranscriptional processing. In preliminary half-life studies, it appears that the two smaller transcripts have half-lives roughly three times longer than those of the larger transcripts, which is not sufficient to account for the disparity in the amounts of the two sets of transcripts under fully induced conditions. Therefore, it seems likely that preferential termination occurs in the *ureCE* intergenic region, which gives rise to a higher proportion of *ureIABC* and *ureABC* transcripts compared with *ureIABCEFGD* and *ureABCEFGD* transcripts.

Localization of *PureI*. Based on the results obtained from Northern blot analyses and the fact that *ureI* is the 5′-most gene in the *ure* cluster, it was hypothesized that *PureI* was differentially regulated in response to the environmental pH and carbohydrate availability. To map the putative location of *PureI*, the 5′ end of the transcript was determined by primer extension analysis. Total cellular RNAs from cells growing at steady state in continuous culture with a dilution rate of 0.3 h^{-1} (generation time ≅ 2.3 h), at pH 7.0 or 5.5 under carbohydrate-limiting conditions (10 mM fructose) or at pH 5.5 under excess carbohydrate conditions (200 mM fructose), were isolated. Cells were grown for at least 10 generations at any single set of growth parameters before cultures were defined to be at steady state, where the specific growth rate was equal to the dilution rate of the vessel (28). Two primers, containing antisense sequences of *ureI*, were used: primer P*ureI*as-100, 5′-TCAAACCCAAACCACCCG-3′, and primer PMC32-1, 5′-CCCTGTACAAGCTCCAT-3′, were located 106 and 148 bases 3′ to the start codon, respectively (Fig. 2). Products extended from each reaction were analyzed on a 6%

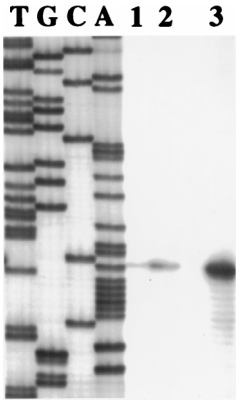

FIG. 4. Primer extension analysis of *PureI*. Total cellular RNA of *S. salivarius* 57.I from steady-state cultures grown in a chemostat were used. Radiolabeled primer PMC32-1 was incubated with the RNA, and the corresponding DNA was synthesized. The same primer was used to prime dideoxy sequencing reactions with plasmid pMC32 as a template. Lanes 1 and 2 show total RNA isolated from pH 7.0 and pH 5.5 cultures, respectively, with 10 mM fructose. Lane 3 shows total RNA isolated from a pH 5.5 culture with 200 mM fructose.

polyacrylamide gel along with a DNA sequencing reaction with the same primer. A signal, 22 bases 5′ to the translational start site of *ureI* (Fig. 4), was consistently observed with either primer under all growth conditions. This transcriptional initiation site could be mapped to a σ^{70}-like promoter sequence located at an appropriate distance (Fig. 2). Furthermore, the intensity of the signal was greater with RNAs isolated from pH 5.5-grown cells than with those from pH 7.0-grown cells. The strongest signal was observed with RNAs isolated from cells grown at pH 5.5 with excess carbohydrate, suggesting that *PureI* is sensitive to both an acidic pH and carbohydrate concentrations.

Based on the results of Northern blot analysis and the $NiCl_2$-dependent urease expression in recombinant streptococcal and *E. coli* strains harboring *ureA* to *-G* (5), the possibility exists that there is a functional promoter 5′ to *ureA* (*PureA*). Our preliminary study using a *PureA-cat* fusion, in which a 400-bp fragment beginning 5′ to the translational start codon of *ureA* was fused to a promoterless chloramphenicol acetyltransferase (CAT) gene (*cat*), also demonstrated a functional but pH-unresponsive streptococcal promoter within this 400-bp region (data not shown). In addition, we found extremely low levels of urease in a strain of *S. salivarius* carrying a polar insertion in *ureI*, where the expression of the *ure* operon should have been derived solely from the activity of *PureA* (data not shown). However, all attempts to localize *PureA* by primer extension have failed. Multiple signals, probably due to readthrough from *PureI*, were observed, making it impossible to determine the location of *PureA*. When these observations are taken together, we cannot rule out the possibility that *PureA* is functional in vivo. However, it is quite clear that *PureA* is not the promoter used for the differential expression of urease in response to environmental signals. Thus, we focused on the analysis of the dominant promoter in the operon *PureI*.

Molecular analysis of the 5′ flanking region of *PureI*. Sequence analysis revealed two inverted repeats, 26 and 83 bases 5′ to the −35 region of *PureI*, with ΔG values of −19 and −15 kcal/mol, respectively (Fig. 2). To determine whether these putative secondary structures could function as *cis*-acting elements in response to environmental signals, two transcriptional fusions to a promoterless *cat* were constructed. The promoterless *cat* fragment containing an *E. coli* RBS was purchased from Pharmacia (Piscataway, N.J.) as a *Hin*dIII fragment. To assist the cloning, sequences recognized by *Sal*I and *Bam*HI were included 5′ to the ATG site of the *cat* fragment (5′-GCG TCGACTGGATCCATGGAGAAAAAAATCACT-3′), and sequences recognized by *Hin*dIII were included in the primer which contains the antisense sequences of the 3′ end of the *cat* sequences (5′-CAAGGATCCAAGCTTCGACGAATT-3′). PCRs were used to amplify the *cat* fragment with the pair of primers described above. The PCR products were initially digested with *Sal*I and *Hin*dIII and then cloned onto *Sal*I- and *Hin*dIII-digested pUC18 to obtain a chimeric plasmid in which the *cat* fragment formed a translational fusion with *lacZ*. The ligation was used to transform *E. coli* DH10B, and selection was carried out to obtain transformants that were resistant to 50 μg of chloramphenicol per ml. The resulting plasmid was designated pCW24. To assist in the construction of pMC68 and pMC71 (see below), the *Hin*dIII site on this *cat* fragment was destroyed by digesting pCW24 with *Hin*dIII, followed by treatment with Klenow fragments and deoxynucleoside triphosphates. The *cat* fragment was then released from pCW24 by *Sal*I digestion. The resulting fragment was subsequently cloned onto *Eco*RV- and *Sal*I-digested pGEM-5Zf(+) (Promega) to generate pCW42.

Because of the proximity of the putative −35 region to the inverted repeat immediately on the 5′ side, it was difficult to design primers for the construction of a deletion derivative completely lacking both inverted repeats. Consequently, the intact promoter (*PureI*) and the deletion derivative lacking 1.5 inverted repeats (*PureI*Δ100) were amplified by PCRs with the primer pair P*ureI*as and P*ureI*s and the primer pair P*ureI*as and P*ureI*de12, respectively (Fig. 2). To facilitate the construction with the promoterless *cat*, a *Bam*HI site was included immediately 5′ to the ATG site of *ureI* in primer P*ureI*as, 5′-CACC TAACATGGATCCCTCCTAAG-3′. Primers P*ureI*s (5′-GGC GACAATCAGTCCCTTAAT-3′) and P*ureI*de12 (5′-TAAGC TTGACTAATATGTAAATG-3′), containing a *Hin*dIII site for cloning, were located 535 and 80 bases 5′ to the ATG site of *ureI*, respectively. The PCR products of *PureI* and *PureI*Δ100 were initially cloned onto pCRII (Invitrogen, Carlsbad, Calif.), and the nucleotide sequences were determined. The correct products were then cloned onto *Bam*HI- and *Hin*cII-digested pCW42, selecting for resistance to chloramphenicol. Because of the lack of an alternative integration vector, a 2.0-kbp fragment (Fig. 1) located 5′ to the inverted repeats was ligated to both constructs at the *Hin*dIII site to generate pMC68 and pMC71, respectively. To facilitate the integration of the reporter constructs into the *S. salivarius* 57.I chromosome, the *ureI* promoter-*cat* fusions were then released from pMC68 and pMC71 and subcloned onto pSF143 (29) in *E. coli*, selecting for transformants resistant to 5 μg of tetracycline per ml and 50 μg of chloramphenicol per ml. The resulting plasmids were designated pCW45 and pMC77, respectively.

Both plasmids were introduced into *S. salivarius* by electroporation according to the guidelines of Caparon and Scott (3) with the following modifications. Briefly, an overnight culture of *S. salivarius* 57.I grown in Todd-Hewitt broth (Difco) and containing 0.2% yeast extract (THY) and 300 mM L-threonine was diluted 1:20 in fresh medium containing the same concentration of L-threonine. Cultures were incubated at 37°C in a 5% CO_2 atmosphere until the optical density at 600 nm reached 0.2. Cells were kept on ice for 10 min prior to harvesting by centrifugation at 4°C. Cells were washed twice with an equal volume of ice-cold electroporation medium (272 mM glucose, 1 mM $MgCl_2$ [pH 6.5]) and then resuspended in 1/15 of the original culture volume in ice-cold electroporation medium. Concentrated cell suspensions were kept on ice for at least 45 min prior to electroporation. Aliquots (40 μl) of the cells were mixed with 200 ng of plasmid DNA and then trans-

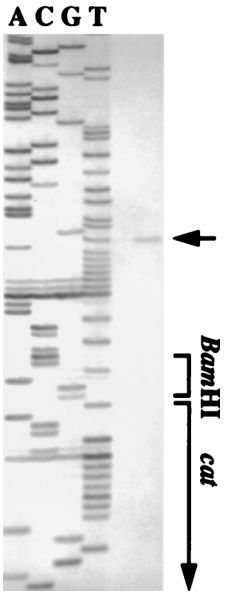

FIG. 5. Primer extension analysis of *PureI*Δ100CAT. Total cellular RNA of *S. salivarius PureI*Δ100CAT from a steady-state culture grown at pH 5.5 with 20 mM glucose was isolated, and the radiolabeled primer *cat* (5′-AATGCCTCAA AATGT-3′) was used. The DNA sequences were derived from pMC71 with the same primer.

ferred into a chilled 0.1-cm Gene Pulser electroporation cuvette. Negative controls included cells with no added DNA. Electroporations were carried out at 1.8 kV, 25 μF, and 200 Ω. Cuvettes, containing cells and DNA, were kept on ice for 2 min after electroporation. The cell suspensions were subsequently recovered with 1 ml of THY plus 10 mM glucose at 37°C in a 5% CO_2 atmosphere for 2 h. Cells were then concentrated and plated on BHI agar supplemented with 3 μg of tetracycline per ml. All plates were incubated at 37°C in a 5% CO_2 atmosphere overnight. Colonies were usually visible in 18 to 24 h. The tetracycline-resistant transformants, strains *PureI*CAT and *PureI*Δ100CAT, were further confirmed by Southern blot analysis with a tetracycline-specific probe (data not shown). Because of the nature of single-cross integration, both strains were predicted to possess a wild-type promoter 5′ to the *ure* cluster and a full-length promoter, or the deletion derivative, 5′ to *cat*. To confirm the presence and configuration of the constructs, a primer located 100 bases 3′ to the ATG and containing the antisense sequences of *cat* and a primer located 5′ to the *Hin*dIII site on the wild-type chromosome were used to amplify the chromosomal region of strains *PureI*CAT and *PureI*Δ100CAT. The PCR products were subsequently cloned onto pCRII and subjected to sequence analysis to confirm that the junction of the promoter region and the *cat* gene was intact and to ensure that the deletion of the inverted repeats 5′ to *cat* had occurred when desired.

To be certain that the expression observed in *PureI*Δ100CAT was due only to *PureI* and not to promoters 5′ to the deleted region, the transcriptional initiation site for the *cat* gene was determined in *PureI*Δ100CAT by primer extension analysis with a primer 100 bases 3′ to the translational start site of *cat* (Fig. 5). The results indicated that the transcriptional initiation site matched the initiation site used in the wild type. The expression of *cat* in both strains was determined in chemostat-grown cultures under different environmental conditions (Table 2). Briefly, cells were harvested and washed once with an equal volume of 10 mM Tris, pH 7.8, and then resuspended in 1/40 of the original culture volume in the same buffer. Concentrated cell suspensions were subjected to mechanical disruption in the presence of an equal volume of glass beads (0.1-mm diameter) by homogenization in a Bead Beater for a total of 2 min at 4°C. The concentration of each protein lysate was measured by using a protein assay (Bio-Rad, Hercules, Calif.), based on the method of Bradford. Bovine serum albumin served as the standard. The rates of chloramphenicol acetylation of each protein lysate were quantitated by the method of Shaw (23). Low levels of expression were observed in *PureI*-CAT grown at a neutral pH under carbohydrate-limiting conditions (Glc concentration = 20 mM). Induction, approximately eightfold increases, occurred when the culture became acidic, with the highest levels observed in cells grown at pH 5.5 under excess carbohydrate conditions (approximately 17-fold increases). This result was consistent with previous observations in which the levels of the *ureC*-specific mRNAs were induced to a comparable magnitude under similar growth conditions (4). Interestingly, at a neutral pH the specific activity in *PureI*Δ100-CAT was approximately 60-fold higher than that in *PureI*CAT. Modest induction by an acidic pH was still observed in *PureI*Δ-100CAT but was never more than twofold under carbohydrate limiting conditions. No further induction by excess carbohydrate was observed in *PureI*Δ100CAT. These data indicated that *PureI* was negatively regulated and that the expression of *PureI*CAT was derepressed at an acidic pH. The slight increase in activity by an acidic pH observed in *PureI*Δ100CAT under carbohydrate-limiting conditions may be due to the presence of partial sequences of the inverted repeat, with which the proposed repressor might still weakly interact.

The regulation of *S. salivarius* 57.I urease expression by the environmental pH and carbohydrate availability, but not nitrogen availability, is distinct from previously defined urease control pathways, yet control of urease expression in this manner seems particularly well suited to the environment occupied by oral streptococci. It has been established that oral streptococci experience large and rapid changes in carbohydrate availability and pH. Moreover, it has been documented that ammonia generation from urea protects *S. salivarius* against lethal acidification (24). Logically, then, substantial up-regulation of urease gene expression at a low environmental pH and high carbohydrate concentrations would offer the organism a mechanism to induce synthesis of this protective system under conditions when the enzyme might be needed most for survival. High levels of ureolysis may be especially important for the survival of *S. salivarius* under extreme acidic conditions, as it is considered to be less aciduric than some oral streptococci and lactobacilli. On the other hand, repression of *ure* expression at a neutral pH could avoid overproduction of ammonia and alkalization of the environment, which can also be lethal for ureolytic organisms (6).

In this study, we have completed the cloning and sequence analysis of the *ure* operon. The operon began with *ureI*, followed by the structural genes, *ureABC*, and then the accessory

TABLE 2. Levels of CAT specific activity in chemostat-grown *S. salivarius* 57.I derivatives

Strain	CAT sp act[a]		
	pH 7.0, 20 mM Glc	pH 5.5, 20 mM Glc	pH 5.5, 200 mM Glc
*PureI*CAT	0.23 ± 0.16	1.93 ± 0.10	4.09 ± 1.17
*PureI*Δ100CAT	13.98 ± 0.97	29.88 ± 0.29	19.34 ± 3.18

[a] Values shown are averages from three independent samples. The numbers are expressed as nanomoles of chloramphenicol acetylated per minute per milligram of protein. Negative controls were reactions carried out in the absence of chloramphenicol. Each set of reactions was performed in triplicate.

genes, *ureEFGD*. The function of UreI is not well defined, although it is clearly not required for urease biogenesis, since heterologous expression of the *S. salivarius ureA* to *-D* in streptococcal hosts yields a urease enzyme indistinguishable from that of the parent (5). Notably, we have investigated the possibility that UreI could be involved in a regulatory circuit governing transcription of *ure* genes in response to pH. Using a strain containing a polar insertion in *ureI*, we found that pH responsiveness of *PureI* remained identical to that of the wild type (data not shown), strongly suggesting that the expression of *ureI* is not autogenously regulated, nor is its gene product required for pH-dependent regulation.

The expression of both CAT and urease was induced by an acidic pH and excess carbohydrate in strain *PureI*CAT, in which the transcription of both the *ure* operon and *cat* was driven by a full-length *PureI*. However, the magnitude of increases in CAT level was substantially less than that of urease. The differences are likely attributable to the observation that the half-lives of CAT and of urease in oral streptococci are dramatically different. It has been shown that the *S. salivarius* urease is extremely stable in vivo (26); thus, the activity observed with each growth condition would reflect the combination of the amount of stable mRNA and the accumulation of a stable enzyme. On the other hand, the turnover rate of CAT in oral streptococci appears to be relatively high (30); hence, the level of CAT specific activities would be more closely related to the level of transcription initiation. Consistent with this, the magnitudes of increases in the levels of transcription initiated by *PureI* measured by primer extension (Fig. 4) and in the levels of *ureC*-specific messages quantitated by slot blot analysis (4) most closely parallel the increases in CAT specific activity in response to an acidic pH and excess carbohydrate. It should also be noted that the level of expression observed in *PureI*CAT under acidic pH and excess carbohydrate conditions never reached the level observed in *PureI*Δ100CAT. It is possible that other factors may influence the expression of *PureI*Δ100CAT, which may have been reflected in the apparent higher strength of *PureI*Δ100. However, it is more likely that *PureI* is not completely derepressed under the conditions examined and that perhaps more-acidic conditions are needed to fully derepress the operon.

In summary, we have established that the urease genes of *S. salivarius* constitute an operon, have identified a promoter that is sensitive to both the environmental pH and carbohydrate availability, and have determined that the induction of urease expression by an acidic pH and excess carbohydrate was negatively regulated. Unlike reports of enteric bacterial ureases, which are either constitutively expressed or regulated by an activator, this is the first report to demonstrate negative control of urease expression. Efforts to isolate and characterize the *trans*-acting factors which bind near *PureI* are under way.

Nucleotide sequence accession numbers. The sequence of *ureI* was submitted to GenBank and assigned accession no. AF042344. The sequences of ORF1, -2, and -3 were assigned GenBank accession no. AF043280, AF043281, and AF043282, respectively.

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