cis*-Acting elements that regulate the low-pH-inducible urease operon of *Streptococcus salivarius

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Differential expression of the *Streptococcus salivarius* 57.I urease operon in response to pH is effected by repression of transcription from a proximal promoter, *Purel*. To localize the *cis*-acting elements involved in the regulation of the urease operon, the intact promoter region and its derivatives were generated and fused to a promoterless chloramphenicol acetyltransferase (*cat*) gene. The promoter-*cat* fusions were established in the *lacZ* gene of *S. salivarius* by using a newly constructed integration vector. CAT-specific activities were examined in batch-grown cells at pH 7.5 and 5.5. The results indicated that a 21 bp region immediately 5' to the -35 element was required for efficient repression of *Purel* at neutral pH and that the 39 bp (-57 to -95) 5' to this region contained sequences required for optimal expression of *Purel*. A potential secondary repressor-binding site was tentatively identified further upstream of the -35 element (-96 to -115). To further analyse the *cis*-acting elements, base changes were introduced into two AT-rich repeats within the primary repressor-binding site. One such derivative, *S. salivarius* M1, with five base substitutions immediately 5' to the -35 element, expressed 20-fold more CAT-specific activity at neutral pH than the strain carrying wild-type *Purel*-*cat*. Also, the pH sensitivity of strain M1 was greatly reduced, suggesting that this AT-rich region is crucial for repression of the urease operon. Deletion of three consecutive 15- or 16-base segments from -52 to -96 in the *S. salivarius* M1 background resulted in lower activities compared to strain M1, confirming the presence of sequences required for optimal expression of the operon. All of the *Purel*-*cat* fusions were also integrated into the *gtfG* gene of *Streptococcus gordonii* DL1, a non-ureolytic oral *Streptococcus* sp. Repression of *Purel* was observed at neutral pH in *S. gordonii* and the effects of the various mutations of the repressor-binding site largely paralleled those seen in *S. salivarius*, suggesting that the *cis*-elements may be a target for a global regulatory circuit that controls gene expression in streptococci in response to pH.

Keywords: *cis*-elements, pH regulation, urease gene expression, oral streptococci

INTRODUCTION

Ureases have been isolated and characterized from a variety of prokaryotes (Mobley & Hausinger, 1989; Mobley *et al.*, 1995). Most bacterial ureases consist of three subunits, α , β and γ , encoded by *ureC*, *ureB* and *ureA*, respectively. In addition to the subunit-

encoding genes, the accessory genes *ureD*, *ureE*, *ureF* and *ureG* have been identified in urease gene clusters and have been shown to encode proteins that are essential for the incorporation of Ni²⁺ into the metal centre of the enzyme. A few urease operons also contain *ureI*, which encodes a urea transporter (Weeks *et al.*, 2000; Weeks & Sachs, 2001). Although high degrees of homology exist between bacterial ureases, the mechanisms for control of urease expression vary among prokaryotes (Mobley *et al.*, 1995; Collins & D'Orazio, 1993). For instance, the expression of *Klebsiella* urease

Abbreviations: *cat* and CAT, chloramphenicol acetyltransferase; *Purel*, promoter of the *ure* operon.

genes is activated only under nitrogen-limiting conditions, whereas the expression of *Proteus* urease genes is activated in the presence of urea by UreR, a *ure* operon-specific regulatory protein.

Urea is probably the most abundant nitrogen source in the oral cavity. Urea is secreted in saliva and crevicular fluids at concentrations of 3–10 mM (Golub *et al.*, 1971; Kopstein & Wrong, 1977) and is rapidly hydrolysed by microbial ureases to yield ammonia and CO₂. Ammonia may inhibit the initiation and progression of dental decay by neutralizing acids generated from bacterial glycolysis (Clancy *et al.*, 2000). In addition, the less acidic conditions resulting from ureolysis provide a more favourable growth environment for those bacterial species that do not compete well at low pH with cariogenic organisms. Thus, ureolysis may have a major impact on oral biofilm ecology (Li *et al.*, 2000; Clancy *et al.*, 2000). Consequently, recent studies have begun to explore the molecular architecture and regulation of the ureases of oral bacteria. For example, the urease gene cluster of *Streptococcus salivarius*, one of the most abundant and highly ureolytic oral micro-organisms, is regulated differently than other known bacterial urease gene clusters. In particular, the *S. salivarius* urease genes are induced by acidic pHs and expression of these genes can be markedly enhanced by growing the organism in acidic conditions and in an excess amount of carbohydrate (Chen & Burne, 1996; Weaver *et al.*, 2000). It is known that oral bacteria commonly deal with large and rapid fluctuations in their environment, especially in pH and in the amount and type of carbohydrate available to them (Burne, 1998). It is reasonable to predict that oral organisms that could not appropriately respond to these environmental changes would be at a competitive disadvantage and would not persist in the biofilms colonizing the teeth and soft tissues. Thus, enhanced expression of *S. salivarius* urease in acidic conditions and with excess amounts of carbohydrate is consistent with the role of urease in protection of the organism from acid killing and the ability of the organism to use urea as a primary source of nitrogen (Chen *et al.*, 2000) – both of these factors could provide a strong advantage for this organism to persist in its natural environment.

The *S. salivarius* urease gene cluster (*ure*) is arranged as an operon, beginning with *ureI*, followed by the genes that encode the structural subunits, *ureABC*, and the genes that encode the proteins required for assembly of the active enzyme, *ureEFGD* (Chen *et al.*, 1998b). A σ^{70} -like promoter that is responsible for differential expression of these genes in response to pH changes and carbohydrate concentrations has been localized immediately 5' to *ureI* (*PureI*). Using promoter fusions of *PureI* and a deletion derivative lacking 100 bp 5' to the –35 element to a chloramphenicol acetyltransferase (*cat*) gene, it was found that the expression of *PureI* is negatively regulated (Chen *et al.*, 1998b), with nearly complete repression of expression at neutral pH. However, no ORFs encoding proteins with characteristics of transcriptional regulators could be identified in the

regions flanking the *ure* gene cluster, raising the possibility that the expression of the *ure* operon may be regulated by factor(s) that are not specific to urease. The purpose of this study was to identify the *cis*-acting elements that are responsible for the differential expression of urease and to explore the possibility that the *S. salivarius* urease may be part of a global circuit that controls gene expression in streptococci in response to pH.

METHODS

Bacterial strains, growth conditions and reagents. The bacterial strains and plasmids used in this study are listed in Table 1. *S. salivarius* 57.I, *Streptococcus gordonii* DL1 and the derivatives of both strains were routinely grown in brain heart infusion (BHI; Difco Laboratories) at 37 °C, in a 5% CO₂ atmosphere. Kanamycin was included in the growth medium at 750 µg ml⁻¹ or 250 µg ml⁻¹ for recombinant *S. salivarius* and *S. gordonii* strains, respectively. Recombinant *Escherichia coli* strains were maintained in L broth supplemented, where indicated, with 50 µg kanamycin ml⁻¹ or 25 µg chloramphenicol ml⁻¹. All chemical reagents and antibiotics were obtained from Sigma. To obtain cultures grown under neutral or acidic pH values, cells were cultured to mid-exponential phase in BHI containing 50 mM potassium phosphate buffer (KPB; pH 7.5) or in BHI that had been adjusted to pH 5.5 by the addition of 2 M HCl (BHI/HCl).

DNA manipulations. Genomic DNA from *S. salivarius* 57.I was isolated as described previously (Chen *et al.*, 1996). Plasmid DNA was isolated from *E. coli* by the method of Birnboim & Doly (1979). Plasmid DNA to be used in sequencing reactions was prepared from *E. coli* DH10B by using the QIAprep Spin Plasmid Kit (Qiagen). Cloning, electrophoretic analysis of DNA fragments, Southern-blot analysis and hybridizations were carried out using established protocols (Ausubel *et al.*, 1989). Restriction and DNA-modifying enzymes were purchased from Life Technologies or New England Biolabs.

Construction of integration vectors for *S. salivarius* 57.I and *S. gordonii* DL1. The integration vector pMC195 was constructed as follows (Fig. 1). The *S. salivarius* 57.I *lacZ* gene was first amplified by PCR using *Taq* DNA polymerase with primer pairs homologous to *lacZ* of *Streptococcus thermophilus* A054 (accession no. M63636), as detailed previously (Chen *et al.*, 2002). The PCR product was initially cloned onto pCRII and the identity of the product was confirmed by sequence analysis. The internal 2.45 kbp *Pst*I–*Xba*I fragment of the PCR product was subsequently purified, blunt-ended by T4 polymerase and cloned into pGEM-3Zf(+) that had been digested with *Hind*III and *Eco*RI followed by Klenow treatment to remove the multiple-cloning sequences (MCSs) to generate pMC194. A DNA fragment containing a kanamycin-resistance gene flanked by T4 transcription/translation terminator signals (*Ωkan*) (Perez-Casal *et al.*, 1991) and MCSs from pGEM-7Zf(+) was subsequently cloned onto a unique *Cl*aI site within the PCR product. The resulting plasmid, pMC195, allowed the integration of foreign genes into the *S. salivarius* 57.I chromosome at the *lacZ* locus with the acquisition of a kanamycin-resistant phenotype.

The integration vector pMJB8 was constructed as follows (Fig. 1). The *S. gordonii* DL1 *gtfG* gene was amplified by PCR using Vent DNA polymerases with primer pairs specific for *gtfG* of *S. gordonii* DL1 (accession no. U12643). Primer *gtfG*S

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant phenotype(s)*	Description	Source/reference
Strains			
<i>S. salivarius</i> 57.1	Ure ⁺	Wild-type host	Sissons <i>et al.</i> (1988)
<i>S. gordonii</i> DL1	Ure ⁻	Wild-type host	LeBlanc & Hasell (1976)
<i>Purel</i> - <i>cat</i>	CAT ⁺ , Ure ⁺	Wild-type chromosome containing a single copy of <i>Purel</i> - <i>cat</i> at <i>lacZ</i> † or <i>gtfG</i> † locus	This study
<i>Purel</i> Δ21 <i>cat</i>	CAT ⁺ , Ure ⁺	Wild-type chromosome containing a single copy of <i>Purel</i> Δ21 <i>cat</i> at <i>lacZ</i> or <i>gtfG</i> locus	This study
<i>Purel</i> Δ40 <i>cat</i>	CAT ⁺ , Ure ⁺	Wild-type chromosome containing a single copy of <i>Purel</i> Δ40 <i>cat</i> at <i>lacZ</i> or <i>gtfG</i> locus	This study
<i>Purel</i> Δ60 <i>cat</i>	CAT ⁺ , Ure ⁺	Wild-type chromosome containing a single copy of <i>Purel</i> Δ60 <i>cat</i> at <i>lacZ</i> or <i>gtfG</i> locus	This study
<i>Purel</i> Δ80 <i>cat</i>	CAT ⁺ , Ure ⁺	Wild-type chromosome containing a single copy of <i>Purel</i> Δ80 <i>cat</i> at <i>lacZ</i> or <i>gtfG</i> locus	This study
M1	CAT ⁺ , Ure ⁺	The AT-rich region 2 bp 5' to -35 element in strain <i>Purel</i> - <i>cat</i> chromosome is mutated to TCTAGA	This study
M2	CAT ⁺ , Ure ⁺	The AT-rich region 12 bp 5' to -35 element in strain <i>Purel</i> - <i>cat</i> chromosome is mutated to TCTAGAGA	This study
M3	CAT ⁺ , Ure ⁺	The AT-rich region 12 bp 5' to -35 element in strain <i>Purel</i> - <i>cat</i> chromosome is mutated to AGATCT	This study
M1Δ(-66 to -52)	CAT ⁺ , Ure ⁺	Strain M1 containing a deletion from -66 to -52 5' to <i>Purel</i>	This study
M1Δ(-81 to -67)	CAT ⁺ , Ure ⁺	Strain M1 containing a deletion from -81 to -67 5' to <i>Purel</i>	This study
M1Δ(-96 to -82)	CAT ⁺ , Ure ⁺	Strain M1 containing a deletion from -96 to -82 5' to <i>Purel</i>	This study
Plasmids			
pCRII	Ap ^r , Km ^r	General cloning vector	Invitrogen
pGEM-3Zf(+)	Ap ^r	General cloning vector	Promega
pGEM-7Zf(+)	Ap ^r	General cloning vector	Promega
pCW24	Ap ^r , Cm ^r	pUC18 carrying promoterless <i>cat</i>	Chen <i>et al.</i> (1998b)
pMC63	Ap ^r , Cm ^r	pCW42 carrying <i>Purel</i>	Chen <i>et al.</i> (1998b)
pMC159	Ap ^r	pGEM-3Zf(+) carrying <i>Purel</i> Δ40	This study
pMC160	Ap ^r	pGEM-3Zf(+) carrying <i>Purel</i> Δ60	This study
pMC161	Ap ^r	pGEM-3Zf(+) carrying <i>Purel</i> Δ80	This study
pMC162	Ap ^r	pGEM-3Zf(+) carrying <i>Purel</i> Δ21	This study
pMC194	Ap ^r	pGEM-3Zf(+) carrying a 2.45 kb internal fragment of <i>lacZ</i> from <i>S. salivarius</i> 57.1	This study
pMC195	Ap ^r , Km ^r	Integration vector for <i>S. salivarius</i>	This study
pMJB6	Ap ^r	pGEM-3Zf(+) carrying a 2.55 kb internal fragment of <i>gtfG</i> from <i>S. gordonii</i> DL1	This study
pMJB8	Ap ^r , Km ^r	Integration vector for <i>S. gordonii</i>	This study

* Ure⁺, urease-positive; Ure⁻, urease-negative; CAT⁺, CAT-positive; Ap^r, ampicillin-resistant; Km^r, kanamycin-resistant; Cm^r, chloramphenicol-resistant.

† *lacZ* if introduced into *S. salivarius*; *gtfG* if introduced into *S. gordonii*.

(5'-GCTAATCAAGTGACCAATG-3') contained the nucleotide sequence encoding amino acids 46–51 of GtfG; primer gtfGAS (5'-ACTTGGACATTACTGTAGC-3') contained the sequence complementary to that encoding amino acids 857–863 of GtfG. The 2.55 kbp PCR product was initially cloned into pGEM-3zf(+), as described above, to generate pMJB6. The identity of the PCR product was confirmed by sequence analysis. The *Ωkan* element, as described above, was subsequently cloned into the unique *EcoRV* site within the PCR

product. The resulting plasmid, pMJB8, allowed the integration of foreign genes into the *S. gordonii* DL1 chromosome at the *gtfG* locus with concomitant acquisition of a kanamycin-resistant phenotype.

Construction of *Purel*-*cat* recombinant fusion strains. The wild-type *Purel* and a set of deletions of the 5' flanking regions of *Purel* were generated by PCR using the primers listed in Table 2. Briefly, the 5' portions of *Purel*Δ21*cat*, *Purel*Δ40*cat*, *Purel*Δ60*cat* and *Purel*Δ80*cat* were amplified from the *S.*

Fig. 1. Construction of pMC195 and pMJB8. The internal fragments of *S. salivarius lacZ* and *S. gordonii gtfg* were generated by PCR, as described in the text. The orientations of all of the ORFs in the chimeric plasmids are indicated by arrows. The transcription/translation termination signals of Ω_{kan} are indicated (● with a stalk). The *EcoRI* site within the MCS on pMC195 is not unique. *bla*, β -lactamase.

Table 2. Oligonucleotide primers used to generate *PureI* and its derivatives

Oligomer	Sequences*	Locations and orientations†
pMC43S-4	5'-AGACTAGTCTTTTCGTCGG-3'	-455 to -437; sense
<i>PureI</i> -70ASHincII	5'-GAAATTGTCAACCATTTACATATTAGTC-3'	-45 to -72; anti-sense
<i>PureI</i> -100ASHincII	5'-CATATTAGTCAACCTAACATGTG-3'	-63 to -85; anti-sense
<i>PureI</i> -120ASHincII	5'-CATGTGGTGTGTCAACATGACATTAC-3'	-80 to -105; anti-sense
<i>PureI</i> -140ASHincII	5'-CATTACATTTGTCAACTCTCAATAGG-3'	-100 to -125; anti-sense
<i>PureI</i> Δ (-66 to -52)S	5'-GCTTGACTAAAAATTTCTGA-3'	-76 to -42; sense
<i>PureI</i> Δ (-66 to -52)AS	5'-TCAGAAAATTTTAGTCAAGC-3'	-76 to -42; anti-sense
<i>PureI</i> Δ (-81 to -67)S	5'-TGACACCACATATGTAAATG-3'	-91 to -57; sense
<i>PureI</i> Δ (-81 to -67)AS	5'-CATTTACATATGTGGTGTCA-3'	-91 to -57; anti-sense
<i>PureI</i> Δ (-96 to -82)S	5'-TGTAATGTCATGTTAGCTTG-3'	-106 to -72; sense
<i>PureI</i> Δ (-96 to -82)AS	5'-CAAGCTAACATGACATTACA-3'	-106 to -72; anti-sense
<i>catAS250</i>	5'-TCTTTCATTGCCATACGGA-3'	217 to 236; anti-sense

* Introduced *HincII* site is shown in bold.

† The position of each oligomer is present according to Fig. 2, except for *catAS250*. The position of *catAS250* is based on the translational start site of *cat*. Sense, oligomers contain sequences in the same orientation as the *ure* operon or *cat*; anti-sense, oligomers contain sequences in the opposite orientation of the *ure* operon or *cat*.

salivarius 57.I chromosome by PCR using primer pairs pMC43S-4 + *PureI*-70HincII, pMC43S-4 + *PureI*-100HincII, pMC43S-4 + *PureI*-120HincII and pMC43S-4 + *PureI*-140HincII, respectively. To be noted, primer pMC43S-4 contains a *SpeI* recognition site, and a *HincII* recognition site is present in primers *PureI*-70HincII, *PureI*-100HincII, *PureI*-120HincII and *PureI*-140HincII (Table 2). Subsequently, the PCR products were digested with *SpeI* and *HincII* and then cloned into *XbaI*- and *HincII*-digested pGEM-3Zf(+) to generate plasmids pMC162, pMC159, pMC160 and pMC161, respectively. The 3' portion of all of the deletion derivatives was generated from *HincII* and *SphI* double-digested pMC63 (Chen *et al.*, 1998b), which resulted in a fragment containing the 60 bp region located immediately 5' to the *ureI* start codon (ATG) that had been fused with a promoterless *cat* (Promega). This *HincII*-*SphI* fragment was subsequently cloned into pMC162, pMC159, pMC160 and pMC161 at unique *HincII* and *SphI* sites. The sequence of each construct was confirmed by sequence analysis. The correct promoter-*cat* fusions were subsequently cloned into pMC195 and pMJB8, and the resulting chimeric plasmids were introduced into *S. salivarius* 57.I by electroporation (Chen *et al.*, 1998b) or into *S. gordonii* DL1 by transformation (LeBlanc & Hassell, 1976), respectively. The correct integration of each construct via allelic exchange into *S. salivarius* and into *S. gordonii* was confirmed by Southern-blot analysis using a *lacZ*- and a *gtfG*-specific probe, respectively.

To construct *S. salivarius* M1-based deletion derivatives, recombinant PCRs were employed (Higuchi, 1990). Briefly, the primary products of *PureI*Δ(−66 to −52) were amplified from the chromosome of strain M1 using primer pairs pMC43S-4 + *PureI*Δ(−66 to −52)AS and *PureI*Δ(−66 to −52)S + *cat*AS250. To be noted, primers *PureI*Δ(−66 to −52)AS and *PureI*Δ(−66 to −52)S contain the same sequence and are complementary to each other. Both primers cover 10 bases flanking each end of the −66 to −52 region, which would result in a PCR product with a deletion of the −66 to −52 region. Primer *cat*AS250 contains a complementary sequence that encodes amino acids 73–79 of chloramphenicol acetyltransferase (CAT). The primary PCRs consisted of 5 cycles at a less-stringent annealing temperature (45 °C), followed by 25 cycles at a high-stringent annealing temperature (55 °C). Equimolar quantities of each product were mixed and PCRs were then performed using primers pMC43S-4 + *cat*AS250 under stringent conditions. The final product was digested with *Bam*HI and *SpeI*, to release the mutated promoter region, followed by cloning onto pCW24, a plasmid harbouring a promoterless *cat* (Chen *et al.*, 1998b). DNA sequence analysis was performed to confirm the correct sequence. The correct promoter-*cat* fusion was subsequently cloned onto pMC195 and pMJB8 and integrated into *S. salivarius* and *S. gordonii*, respectively. The integration of the plasmids by double-crossover was confirmed by Southern-blot analysis. Similarly, *PureI*Δ(−81 to −67) was amplified by using primer pairs pMC43S-4 + *PureI*Δ(−81 to −67)AS and *PureI*Δ(−81 to −67)S + *cat*AS250, and *PureI*Δ(−96 to −82) was amplified by using primer pairs pMC43S-4 + *PureI*Δ(−96 to −82)AS and *PureI*Δ(−96 to −82)S + *cat*AS250. Secondary PCRs were then performed with both sets of products by using the primer pair pMC43S-4 + *cat*AS250.

Site-directed mutagenesis. Site-directed mutagenesis of the AT-rich regions 5' to *PureI* was carried out by using the Chameleon double-stranded, site-directed mutagenesis kit (strain M1; Stratagene) or by PCR (strains M2 and M3). Mutagenic oligonucleotides for constructing strains M1 (5'-GCAAAATTTCTGTCTAGACGTTGACATGTG-3') and

M2 (5'-CACATGTCAACGAATTTTCAGATCTCTAGAGCAACATTTAC-3') were designed to create an *XbaI* site (shown in bold) that could be used to screen for clones containing the correct mutations. Oligomer 5'-CACATGTCAACGAATTTTCAGAAGATCTGCAACATTTAC-3' was used to generate strain M3. The sequences of all of the constructs with the desired mutations were confirmed and promoter-*cat* constructs carrying the correct mutations were introduced into *S. salivarius* 57.I or *S. gordonii* DL1, as described above.

CAT assay. Recombinant *S. salivarius* and *S. gordonii* strains were grown in BHI containing 50 mM KPB (pH 7.5) or in BHI/HCl (pH 5.5) to an OD₆₀₀ value of ~0.5. 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 (Biospec Products) for a total of 40 s at 4 °C. The concentration of each protein lysate was measured by using the Bio-Rad Protein Assay based on the method of Bradford (1976). BSA served as the standard. CAT activities were calculated based on the rates of chloramphenicol acetylation, as detailed by Shaw (1979).

RESULTS

Functional analysis of the 5' flanking region of *PureI* in recombinant *S. salivarius* strains

Previous studies using chemostat cultures of recombinant *S. salivarius* promoter fusion strains demonstrated that a deletion of 100 bp 5' to the *PureI* region resulted in higher levels of expression from the promoter at both pH 7.0 and 5.5 compared to wild-type promoter fusions, suggesting that the expression of *PureI* is negatively regulated (Chen *et al.*, 1998b). To localize the potential repressor-binding site(s) involved in the regulation of the *ure* operon, the intact promoter region and a set of deletion derivatives were amplified by PCR and all of the products were fused to a promoterless *cat*. To reliably analyse the effect of each deletion on expression from *PureI*, it was desirable to have stable, single-copy insertions of the gene fusions. Since there were no integration vectors for *S. salivarius*, a new vector (pMC195) was constructed for this organism (see Methods), which allowed the integration of the fusions in single copy in a way that did not perturb the urease locus.

All of the promoter fusions were cloned into pMC195 (Fig. 1) with the *Ωkan* element 5' to the *PureI*-*cat* fusions and integrated into the *S. salivarius* *lacZ* locus. In addition, the *PureI*-*cat* fusions were positioned such that transcription from *PureI* was in either orientation with respect to the *lacZ* gene. Although repression of expression from *PureI* is not as complete in batch culture at neutral pH values as it is in a chemostat, the use of chemostats was not practical for obtaining data from the large number of constructs examined in this study. Thus, we elected to measure *cat* expression in each recombinant strain grown batch-wise in media buffered at pH 7.5 or adjusted to pH 5.5. Importantly, the use of buffered media and batch cultivation in this study

Table 3. CAT-specific activities in recombinant *S. salivarius* and *S. gordonii* strains

Values shown are means $\pm$ SD from three independent samples. The results are expressed as nmol chloramphenicol acetylated min^{-1} (mg total protein) $^{-1}$. Negative controls were reactions carried out in the absence of chloramphenicol. Each set of reactions was performed in triplicate.

<i>PureI</i> -cat derivatives	CAT-specific activity			
	<i>S. salivarius</i> 57.1		<i>S. gordonii</i> DL1	
	pH 7.5	pH 5.5	pH 7.5	pH 5.5
<i>PureI</i> -cat	4.7 $\pm$ 1.1	21.6 $\pm$ 2.3	2.2 $\pm$ 0.2	11.3 $\pm$ 0.2
<i>PureI</i> Δ 21cat	62.8 $\pm$ 17.0	85.9 $\pm$ 6.8	11.2 $\pm$ 1.0	55.7 $\pm$ 3.2
<i>PureI</i> Δ 40cat	16.6 $\pm$ 1.8	31.8 $\pm$ 1.6	3.1 $\pm$ 0.4	14.9 $\pm$ 0.6
<i>PureI</i> Δ 60cat	10.1 $\pm$ 0.6	25.1 $\pm$ 0.7	1.7 $\pm$ 0.3	12.3 $\pm$ 1.6
<i>PureI</i> Δ 80cat	27.6 $\pm$ 2.1	64.3 $\pm$ 9.9	8.1 $\pm$ 0.5	42.7 $\pm$ 2.2

Fig. 2. The nucleotide sequence of *PureI* and the 5' flanking region. The transcription initiation site of *PureI* is indicated with an arrow (+1). The putative -10 and -35 sequences are in bold and the ribosome-binding site (RBS) is in italics. The stem portion of the predicted stem-loop structure is labelled with asterisks. The limits of the deletion derivatives are indicated by the numbers. Strain *PureI* Δ 21cat contains a deletion from -56 to -36, strain *PureI* Δ 40cat contains a deletion from -75 to -36, strain *PureI* Δ 60cat contains a deletion from -95 to -36 and strain *PureI* Δ 80cat contains a deletion from -115 to -36. The AT-rich regions that were subjected to site-directed mutagenesis to generate strains M1, M2 and M3 are shaded; the mutated sequences are also shown. The locations of the 15/16 bp deletions in strain M1 are indicated below the sequences. The sequence is marked with dots every 20 bases.

allowed for observation of significant differences in the expression from the *PureI* promoter at the two pH values tested. The results indicated that the orientation of the promoter fusions with respect to the *lacZ* gene did not significantly affect the levels of CAT activity; hence, only the results from fusion strains with *PureI* transcribed in the same orientation as the interrupted *lacZ* gene are presented here (Table 3). In agreement with previous observations, wild-type *PureI* was sensitive to environmental pH, with higher levels of expression observed in cells grown at pH 5.5, as compared to cells grown at pH 7.5. The recombinant strain containing a 21 bp deletion immediately 5' to the -35 element (strain *PureI* Δ 21cat) expressed more than 10-fold and fourfold higher levels of CAT than the wild-type promoter at pH 7.5 and 5.5, respectively, suggesting the presence of target site(s) within this region for repressor(s) binding. When an additional 19 bp (strain *PureI* Δ 40cat) or 39 bp (strain *PureI* Δ 60cat) immediately

5' to the 21 bp region were deleted, the levels of CAT expression in the resulting recombinant strains were reduced compared to those observed in strain *PureI* Δ 21cat at both pH values, suggesting the presence of sequences required for optimal expression within this 39 bp region. However, when 80 bp were deleted immediately 5' to the -35 element (*PureI* Δ 80cat), the CAT activities of the resulting recombinant strain were greater than the activities of the wild-type *PureI*-cat strain and strains *PureI* Δ 40cat and *PureI* Δ 60cat, but they were less than the activities of strain *PureI* Δ 21cat at both pH values, raising the possibility that the region located between positions -96 and -115 could also contribute to repression of the urease promoter. Even though marked derepression of expression occurred in all of the deletion derivatives at neutral pH, some sensitivity to pH was consistently retained in the deletion derivatives, albeit at variable levels and at levels below that seen for the intact *PureI* promoter region.

Table 4. Functional analysis of the AT-rich regions and the positively regulated region in recombinant *S. salivarius* and *S. gordonii* strains

Values shown are means $\pm$ SD from three independent samples. The results are expressed as nmol chloramphenicol acetylated min⁻¹ (mg total protein)⁻¹. Negative controls were reactions carried out in the absence of chloramphenicol. Each set of reactions was performed in triplicate.

<i>PureI</i> - <i>cat</i> derivatives	CAT-specific activity			
	<i>S. salivarius</i> 57.1		<i>S. gordonii</i> DL1	
	pH 7.5	pH 5.5	pH 7.5	pH 5.5
M1	79.5 $\pm$ 23.3	148.7 $\pm$ 44.7	5.5 $\pm$ 0.1	32.9 $\pm$ 0.9
M2	20.0 $\pm$ 2.1	49.4 $\pm$ 14.8	1.5 $\pm$ 0.4	12.5 $\pm$ 0.6
M3	46.5 $\pm$ 8.4	127.0 $\pm$ 25.2	2.9 $\pm$ 0.4	17.6 $\pm$ 2.0
M1 Δ (-66 to -52)	34.9 $\pm$ 5.6	83.1 $\pm$ 8.2	4.7 $\pm$ 0.3	28.9 $\pm$ 3.3
M1 Δ (-81 to -67)	43.7 $\pm$ 12.1	94.1 $\pm$ 6.1	5.1 $\pm$ 0.6	29.9 $\pm$ 5.2
M1 Δ (-96 to -82)	42.9 $\pm$ 5.0	90.9 $\pm$ 10.7	4.8 $\pm$ 0.8	27.6 $\pm$ 3.1

Roles of the AT-rich sequences within the 21 bp region immediately 5' to the -35 element in the repression of *PureI* in *S. salivarius*

Based on the results of the deletion analyses performed in this study, it appeared that the 21 bp region immediately 5' to the -35 element was the primary target for efficient repression of *PureI*. Sequence analysis revealed the presence of two AT-rich direct-repeats within this region (Fig. 2). To analyse the potential roles of these AT-rich sequences in urease gene regulation, mutations were introduced into the 21 bp region by site-directed mutagenesis or by PCR (see Methods). The mutated promoter regions were subsequently fused to *cat* and integrated into wild-type *S. salivarius* 57.1 in *lacZ*. *S. salivarius* M1, in which the AT-rich region located 2 bp 5' to the -35 element was mutated from AAAATT to TCTAGA (Fig. 2), expressed similar levels of CAT activity to those observed in *S. salivarius* *PureI* Δ 21*cat* at pH 7.5 and 5.5 (Table 4). These results suggested that this AT-rich region was required for the efficient repression of *PureI* expression. *S. salivarius* M2 and M3 contained mutations in the AT-rich region located 12 bp 5' to the -35 element (Fig. 2). Mutation of the 5' AAAATT sequence in the 21 bp region by the introduction of an additional two bases (TCTAGAGA) (strain M2) or from AAAATT to AGATCT (strain M3) also resulted in derepression of *PureI*, although the magnitude of the derepression was less than that seen in strains M1 or *PureI* Δ 21*cat* (Fig. 2). Thus, both AT-rich regions within the 21 bp region participate in the repression of *PureI* at neutral pH (Table 4), but not to an equivalent extent.

Identification of sequences required for the optimal expression of *PureI* in *S. salivarius*

Based on the observation that lower levels of CAT activity were measurable in *S. salivarius* strains harbouring the *PureI* Δ 40*cat* and *PureI* Δ 60*cat* promoter fusions than in the *PureI* Δ 21*cat* strain, it was hypo-

thesized that the 39 bp region located immediately 5' to the primary repressor-binding site (the 21 bp region immediately 5' to the -35 element) may contain sequences needed for optimal expression from *PureI*. However, any effects of mutating only these regions when the 21 bp region was intact would be masked by repression. Therefore, to locate potential positive *cis*-acting site(s), three consecutive 15- or 16-base deletions in the region 5' to the 21 bp region were generated by PCR using the M1 fusion as the base amplification target, creating three derivatives in which efficient repression of *PureI* was relieved. All constructs were subsequently fused to the promoterless *cat* gene and integrated into *S. salivarius*. All three deletion derivatives of the M1 promoter fusion expressed lower levels of CAT activity than strain M1 (Table 4), demonstrating the presence of sites within the 39 bp region 5' to the primary repressor-binding site that were necessary for optimal *PureI*-directed transcription.

Functional analysis of the *cis*-elements of *PureI* in an urease-negative host

To determine whether a global circuit might be involved in the regulation of *PureI* expression in response to environmental signals, and principally to pH, the expression of the various *PureI* derivatives was evaluated in a non-ureolytic *Streptococcus* sp., *S. gordonii* DL1. The urease-negative phenotype is an established phenotype for *S. gordonii* (Whiley & Beighton, 1998). To establish the *PureI*-*cat* fusions in *S. gordonii*, we constructed a vector (pMJB8; Fig. 1) that allowed the integration of foreign DNA into the *gtfG* locus by allelic exchange. All promoter-*cat* fusions were cloned into pMJB8 with the Ω *kan* element 5' to *PureI*-*cat* and with the *PureI* fusions transcribed either in the same or the opposite orientation with respect to *gtfG*. The CAT activities of all of the recombinant *PureI*-*cat* strains were measured in batch-grown cells at pH 7.5 and 5.5. The orientation of *PureI* fusions in relation to the *gtfG*

gene did not influence the results significantly, so only data from constructs carrying fusions in the same orientation as *gtfG* are presented. Four primary findings arose from the use of *S. gordonii* as the urease-negative host. First, the pH sensitivity of expression of *Purel-cat* was conserved in an organism lacking urease genes, with a fivefold increase in CAT activity in cells grown at pH 5.5 versus those grown at pH 7.5 (Table 3). Second, the patterns of regulation in recombinant *S. gordonii* strains harbouring the various deletion derivatives of *Purel-cat* essentially paralleled those observed in recombinant *S. salivarius* strains, although lower levels of activity were consistently observed in *S. gordonii* with each of the deletion constructs (Table 3). Third, *S. gordonii* M1 expressed higher levels of CAT activities than *S. gordonii* carrying the wild-type *Purel-cat* at pH 7.5 and 5.5, although the levels were not as high as those observed in *S. gordonii* *Purel*Δ21*cat* (Table 4). Additionally, mutations in the AT-rich region further upstream of the -35 element (strains M2 and M3) did not significantly alter the expression of *Purel* at either pH value tested, when compared to *S. gordonii* *Purel-cat*. These results indicated that although the primary target for the negative regulation of *Purel* was recognized by the regulatory machinery of *S. gordonii*, there appear to be differences in the binding specificity of the repressor-binding site between *S. salivarius* and *S. gordonii*. Finally, when the functions of the potential positive *cis*-element(s) located 5' to the primary repressor-binding site were also evaluated in the *S. gordonii* M1 background (Table 4), it was found that all three deletion derivatives expressed levels of CAT activities comparable to the parent M1 strain at pH 7.5 and 5.5. Therefore, the sequences 5' to the primary repressor-binding site did not appear to enhance transcription from *Purel* as was observed in *S. salivarius*.

DISCUSSION

The differential expression of *S. salivarius* urease genes is predominantly regulated at the transcriptional level from the *Purel* promoter, and the *Purel* promoter is the only urease promoter known to be sensitive to pH, carbohydrate availability and growth rate (Chen & Burne, 1996; Chen *et al.*, 1998b). The *ure* cluster and the *Purel* promoter present an ideal model for the study of the molecular basis for gene regulation by the environmental factors known to be most important in governing the pathogenic potential of supragingival biofilms. In this study, we have identified the key *cis*-acting elements critical for the control of *Purel* transcription and have disclosed evidence that implicates urease as part of a global regulatory circuit that may be conserved in other streptococci.

A practical by-product of this study was the generation of two integration vectors that were designed specifically for use in the study of gene regulation in two of the most abundant commensal oral streptococci, *S. salivarius* and *S. gordonii*. The organization of the integration vectors permits simple cloning and stable integration of gene fusions or heterologous DNA into these oral strep-

tococci, and the placement of strong transcription/translation terminator signals (the Ω element of the *Ωkan* cassette) between the cloning site and the upstream gene of the host assures that transcriptional read-through will not affect results obtained in gene-fusion studies or adversely influence the expression of heterologous genes. The high degree of homology between the *lacZ* locus of *S. salivarius* and that of *S. thermophilus* (93% identity) may allow one to use pMC195 as an integration vector in *S. thermophilus* as well, although we have not yet determined whether this is possible. A recent genome-sequencing effort has been undertaken for *S. gordonii* (<http://tigrblast.tigr.org/ufmg/>), and the integration plasmid detailed in this study should prove highly valuable for future regulation and virulence studies involving this organism.

The results of deletion analyses provided consistent evidence that transcription from *Purel* is repressed at neutral pH through *cis*-acting elements located immediately 5' to the -35 element of the promoter. However, there is always some concern with deletion studies that the observed effects on regulation do not result directly from the removal of sequences, but rather from the juxtapositioning of non-native sequences closer to the promoter element(s). The use of site-directed mutagenesis obviated this concern, providing strong evidence that the two AT-rich direct-repeats within the 21 bp region 5' to the -35 element were target sites for repression of the *ure* operon. Although this 21 bp region is clearly the primary target site for repression, we cannot exclude the possibility that a secondary repressor-binding site may exist for the *ure* operon. Specifically, the potential presence of such an element became apparent in the *Purel*Δ80*cat* deletion derivative, which showed a rebound in *cat* expression over the *Purel*Δ40*cat* and *Purel*Δ60*cat* constructs. Thus, at this stage, we propose that the primary control of urease expression occurs through repressor binding in the 21 bp region 5' to the -35 element, and possibly at nucleotides -96 to -115 as well. It is also noteworthy that there is no obvious sequence conservation between the 21 bp region and the -96 to -115 region.

Another consistent observation in this study was that we were never able to completely eliminate the pH sensitivity of *Purel* expression with any of the constructs used in this study. The simplest interpretation of this observation is that the repressor-binding site may actually overlap with the -35 sequence and, regardless of what occurs upstream of the promoter, there will always be some residual repressor binding. Isolation of the repressor and subsequent footprint analysis should help to resolve whether this idea is valid. Alternatively, additional sequences located 3' to the -35 element may also be involved in the regulation of urease expression in a pH-dependent fashion. Since we utilized the cognate urease ribosome-binding site in all of our fusions, this could also reflect some level of translational control in response to pH. Translational control of gene expression is not atypical for growth-rate-dependent expression (Carter-Meunchau & Wolfe, 1989), but we are not

aware of any cases where pH-dependent gene expression is modulated translationally. Notably, the urease mRNA of *Helicobacter pylori* is more stable at low pH (Akada *et al.*, 2000). However, our previous analyses of the urease mRNA levels in *S. salivarius* grown under various conditions would indicate that the expression of the *ure* operon is mainly controlled by transcriptional initiation. A definitive demonstration of the pathways involved in expression of the *ure* operon, other than that involved in the control of transcriptional initiation of the urease genes, will be performed when the urease repressor gene is isolated.

It was also of interest that deletion of the DNA sequences located within 39 bp 5' of the 21 bp element resulted in decreased expression from *Purel*. Again, the possibility that these results were obtained because of a change in spacing or moving of non-native sequences closer to the promoter was diminished, but not eliminated, by the findings that the M1 deletion derivatives also showed diminished expression from the constructs lacking the -52 to -96 sequences. Based on computer predictions, we could locate a potential stem-loop structure ($\Delta G^\circ = -19.0 \text{ kcal mol}^{-1}$) from -62 to -84 (Fig. 2), but the role of this secondary structure in regulation is not clear at this point. Furthermore, the use of computer algorithms did not reveal the presence of any sequences within this region that were bound by known transcriptional activators.

Previous studies using continuous chemostat cultures have shown that carbohydrate availability and the sugar:phosphoenolpyruvate phosphotransferase system are involved in the regulation of urease expression (Chen & Burne, 1996; Chen *et al.*, 1998a; Weaver *et al.*, 2000); these studies linked a system capable of sensing carbohydrate source and availability with induction of the urease genes, although pH was still the dominant influence. To help clarify whether the influences of pH and carbohydrate are exerted through a single regulator or through two separate regulatory proteins in *S. salivarius*, we also measured CAT activity in chemostat-grown *S. salivarius* *Purel-cat*, *Purel* Δ 21*cat* and M1 at pH 7.0 and 5.5, with limiting or excess amounts of carbohydrate (data not shown). The idea, in this case, was that if the effects of carbohydrate were exerted through a different *cis*-acting site than that to which the repressor bound, pH sensitivity would be lost but carbohydrate sensitivity would be retained. We found that, unlike with the intact *Purel* promoter, carbohydrate availability did not influence the expression of *Purel* in the strains that lacked the primary repressor-binding site, regardless of the growth pH, suggesting that pH and carbohydrate regulation of urease expression do not operate independently. In addition, we also found that the pattern of *cat* expression in each strain when grown under both pH values paralleled that observed with batch-grown cultures, indicating that the batch-culture system was adequate for this study.

Based on sequence analysis we could not identify any ORF that could potentially encode a DNA-binding

protein in the flanking sequences of the *ure* operon, nor could we identify any known regulatory sequences around the *Purel* region based on BLAST searches against the GenBank database. The lack of a linked regulatory gene, coupled with the fact that urease transcription is rapidly induced at low pH (Burne & Chen, 1998) and, thus, could be part of an acid-shock or acid-tolerance response, led us to postulate that perhaps urease was controlled as part of a global regulon for the low-pH induction of genes. To test this idea, we introduced the *Purel-cat* fusions into the urease-negative oral *Streptococcus* sp. *S. gordonii*. The most notable findings from this experiment included that the *Purel-cat* fusions were pH-sensitive in *S. gordonii* and that the target sequences for repression that were recognized by *S. salivarius* were also recognized by *S. gordonii*. However, there were two notable differences in the regulation of *Purel* in *S. gordonii* compared to its regulation in *S. salivarius*. The first was the lack of involvement of the upstream sequences in positive regulation of the urease promoter in the M1 background. This may simply reflect an absence of, or an absence of recognition by, accessory factors in *S. gordonii* that fine-tune urease expression in *S. salivarius*. There was also some slight variability in the behaviour of the M2 and M3 mutants in *S. gordonii* when compared with the behaviour of these targeted mutations in *S. salivarius*, possibly due to slight variations in the target-site specificity of the *trans*-acting factor that governs expression in response to pH. Notwithstanding these facts, in all cases the behaviour of the derivatives with a mutated primary repressor-binding site in an organism that lacks a urease gene cluster (i.e. *S. gordonii*) paralleled the behaviour of the same mutants in the parent *S. salivarius*. Interestingly, we have also noted that the urease promoter is sensitive to pH in *Streptococcus mutans*, but the magnitude of induction at low pH is only about twofold (Y.-H. Li & R. A. Burne, unpublished data). Collectively, these data support that urease may be the target for global regulators that control gene expression in response to environmental pH and possibly carbohydrate availability and growth rate as well. Thus, the study of the low-pH-inducible urease operon is highly relevant to the development of a thorough understanding of how abundant oral streptococci regulate gene expression in response to those environmental factors that have been shown to be crucial for the development of caries and other common oral health maladies.

ACKNOWLEDGEMENTS

We thank J. A. C. Lemos for helpful discussion. This work was supported by Public Health Service grant DE10362 from the National Institute for Dental Research to R. A. B.

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Received 20 March 2002; revised 28 May 2002; accepted 15 July 2002.