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Streptococcus salivarius Urease: Genetic and Biochemical Characterization and Expression in a Dental Plaque Streptococcus

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The hydrolysis of urea by urease enzymes of oral bacteria is believed to have a major impact on oral microbial ecology and to be intimately involved in oral health and diseases. To begin to understand the biochemistry and genetics of oral ureolysis, a study of the urease of *Streptococcus salivarius*, a highly ureolytic organism which is present in large numbers on the soft tissues of the oral cavity, has been initiated. By using as a probe a 0.6-kbp internal fragment of the *S. salivarius* 57.I *ureC* gene, two clones from subgenomic libraries of *S. salivarius* 57.I in an *Escherichia coli* plasmid vector were identified. Nucleotide sequence analysis revealed the presence of one partial and six complete open reading frames which were most homologous to *ureIAB-CEFGD* of other ureolytic bacteria. Plasmid clones were generated to construct a complete gene cluster and used to transform *E. coli* and *Streptococcus gordonii* DL1, a nonureolytic, dental plaque microorganism. The recombinant organisms expressed high levels of urease activity when the growth medium was supplemented with $NiCl_2$. The urease enzyme was purified from *E. coli*, and its biochemical properties were compared with those of the urease produced by *S. salivarius* and those of the urease produced by *S. gordonii* carrying the plasmid-borne *ure* genes. In all cases, the enzyme had a K_m of 3.5 to 4.1 mM, a pH optimum near 7.0, and a temperature optimum near 60°C. *S. gordonii* carrying the urease genes was then demonstrated to have a significant capacity to temper glycolytic acidification in vitro in the presence of concentrations of urea commonly found in the oral cavity. The ability to genetically engineer plaque bacteria that can modulate environmental pH through ureolysis will open the way to using recombinant ureolytic organisms to test hypotheses regarding the role of oral ureolysis in dental caries, calculus formation, and periodontal diseases. Such recombinant organisms may eventually prove useful for controlling dental caries by replacement therapy.

Ureases (urea amidohydrolases [EC 3.5.1.5]), which catalyze the hydrolysis of urea to ammonia and carbon dioxide, are multisubunit enzymes that require nickel ions for full enzymatic function (15, 48; reviewed in references 33 and 34). The majority of bacterial ureases are similar to that of *Klebsiella aerogenes*, which consists of three subunits, α, β, and γ, encoded by the *ureC*, *ureB*, and *ureA* genes, respectively (35), although exceptions exist (26). Overall, a high degree of homology exists among the prokaryotic enzymes (11, 14, 33), and the predicted amino acid sequences of microbial urease subunits are homologous to the urease enzyme of jack beans (11, 23, 47).

It is also known that in addition to the subunits of the urease enzyme, accessory proteins are required in vivo for the expression of active ureases by bacteria (11, 13, 28, 34, 35, 37–39). The genes encoding these proteins have been found in various operonic arrangements adjacent to the structural genes. Existing information indicates that the protein products of the accessory genes (*ureDEFG*) are involved in the incorporation of nickel ions into the urease apoenzyme (37, 39). Evidence that supports the theory that optimal expression of urease enzymes by bacteria may require that the organisms produce high-affinity nickel transport systems, since nickel is present in only trace amounts in normal biological and natural ecosystems, is accumulating. In many cases, such high-affinity nickel transporters in ureolytic bacteria have been functionally identified and shown to enhance urease activity (17, 32, 49).

Our interests in bacterial ureases arise from a number of observations that support the involvement of urea and ureolysis in oral health and oral microbial ecology. Urea enters the mouth in saliva and crevicular fluids at concentrations of 3 to 10 mM in healthy individuals (19). Ammonia production from urea hydrolysis is a primary source of amino nitrogen and of alkali in the oral cavity. Urea metabolism is thought to inhibit the initiation and progression of dental caries (24, 40). Ureolysis can moderate glycolytic acidification of dental plaque (24) and therefore is proposed to inhibit the initiation and progression of dental caries by neutralizing plaque acids and by fostering an environment less conducive to the emergence of acidogenic and aciduric species (7, 8). The elevated pH values observed in dental plaques as a result of metabolism of urea may also enhance the deposition of minerals, which is necessary for and characteristic of the formation of calculus (30). Likewise, urea enters the subgingival environment in saliva and gingival crevicular fluid at millimolar concentrations, and there it is rapidly metabolized to ammonia and CO_2 (19, 25). The resulting alkaline conditions can enhance mineral deposition on tooth root surfaces, and the high ammonia concentrations can have deleterious effects on host cells (21), such as fibroblasts and polymorphonuclear leukocytes. Thus, ureolysis may contribute to the development of and the tissue destruction seen in cases of gingivitis and periodontitis (21).

Despite the abundance of urea in oral secretions; the high level of urease-specific activity in dental plaques, on soft tis-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype[a]	Description[b]	Source or reference
Strains			
Escherichia coli			
DH10B	Urease −	Host for general cloning	LTI[c]
MC10	Cm^r urease −	DH10B carrying pMC10	This study
MC11	Cm^r urease −	DH10B carrying pMC11	This study
MC12	Cm^r urease +	DH10B carrying pMC12	This study
Streptococcus salivarius 57.I	Urease +	Source for *ure* gene cluster	45 C. Sissons
Streptococcus gordonii			
DL1	Sp^s urease −		D. LeBlanc
DL278	Sp^r urease −	DL1 carrying pDL278	25
MC17	Sp^r urease +	DL1 carrying pMC17	This study
Plasmids			
pSU21	Cm^r	Moderate-copy-number *E. coli* vector	3
pDL278	Sp^r	*E. coli*-streptococcus shuttle vector	16
pMC10	Cm^r	pSU21 *ureI*ABC**	This study
pMC11	Cm^r	pSU21 *ureC*EFGD*	This study
pMC12	Cm^r urease +	pSU21 *ureI*ABCEFGD*	This study
pMC17	Sp^r urease +	pDL278 *ureI*ABCEFGD*	This study

[a] Urease −, urease negative; urease +, urease positive.
[b] For the *ureI* and *ureC* genes, an asterisk indicates that only a partial copy of the gene was carried on the plasmid. See Fig. 1 and the text for more detailed descriptions of recombinant plasmids pMC10, pMC11, pMC12, and pMC17.
[c] LTI, Life Technologies.

sues, and in saliva; and the critical roles that urea and oral ureolysis are thought to play in oral health and disease, little is known about ureolytic microorganisms in the oral cavity (6, 41, 44) and there is no molecular or biochemical information available about urease enzymes of oral bacteria. Among the species of oral bacteria that have been identified as ureolytic, *Streptococcus salivarius* is routinely found in large numbers on the soft tissues of the mouth (7) and can produce high levels of urease (44). This study describes the molecular characterization of the urease genes, characteristics of the expression in *Escherichia coli* and in a dental plaque streptococcus of the recombinant enzyme, the development of a purification protocol for the cloned urease, and an examination of some relevant biochemical properties of the enzyme of *S. salivarius* 57.I, an oral isolate possessing a stable urease phenotype. Finally, the ability of a dental plaque microorganism that is normally nonureolytic to temper glycolytic acidification in vitro in the presence of urea when carrying the cloned urease genes is examined.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and reagents. The bacterial strains and plasmids used in this study are listed in Table 1. Oral streptococci were maintained on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.). Plasmid-bearing *Streptococcus gordonii* organisms were selected and maintained on BHI agar that was supplemented with 250 μg of spectinomycin per ml. *E. coli* strains were maintained on L (42) agar and were grown at 37°C with aeration in L broth. Where indicated, media were supplemented with spectinomycin (100 μg/ml), chloramphenicol (25 μg/ml), or ampicillin (100 μg/ml). When recombinant organisms were analyzed for urease expression, *E. coli* strains were grown in M9 (42) containing 0.1% Casamino Acids and chloramphenicol (10 μg/ml). Plasmid-bearing *S. gordonii* strains were grown in TY (9) containing 10 mM glucose or 250 μg of spectinomycin per ml. All chemical reagents and antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.).

DNA manipulations. Genomic DNA from *S. salivarius* 57.I was isolated as follows. Cells were grown to mid-exponential phase in BHI broth supplemented with 20 mM D,L-threonine and 10 mM glucose. Cells were washed once with 10 mM sodium phosphate buffer (pH 7.0) and incubated with lysozyme (0.2 mg/ml of culture volume) and mutanolysin (0.1 U/ml of culture volume) for 30 min at 37°C. Cells were lysed with 1% sodium dodecyl sulfate (SDS) and proteinase K (50 μg/ml). DNA was then isolated from the lysate as previously described (2). Plasmid DNA was isolated from *S. gordonii* DL1 by the method of Anderson and McKay (1) and from *E. coli* by the method of Birnboim and Doly (5). When desired, plasmid DNA was further purified by centrifugation to equilibrium in cesium chloride-ethidium bromide (42). Plasmid DNA to be used in sequencing reactions was prepared from *E. coli* DH10B by using the QIAprep Spin Plasmid Kit (Qiagen, Inc., Chatsworth, Calif.). Restriction and DNA-modifying enzymes were obtained from Life Technologies (Bethesda, Md.) or from U.S. Biochemicals (Cleveland, Ohio).

For preparation of a subgenomic library of *S. salivarius* 57.I, total chromosomal DNA from this organism was partially digested with *Sau*3A under conditions enriching for DNA fragments of approximately 5 kbp. The resulting DNA fragments were separated on a 0.8% Tris-acetate-EDTA agarose gel (42), and DNA fragments ranging in length from 2 to 3 kbp and from 3 to 7 kbp were isolated separately by using an S & S Elu-Quik DNA Purification Kit (Schleicher & Schuell, Keene, N.H.). The isolated DNA fragments were ligated onto the *Bam*HI-digested, phosphatase-treated (calf intestinal alkaline phosphatase) plasmid vector pSU21 (3) by using T4 DNA ligase. Positive transformants were detected by colony hybridization (42) using an internal fragment of the *ureC* gene of *S. salivarius* 57.I as a probe (10) under stringent hybridization conditions.

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain termination method (43) as modified for use with Sequenase (U.S. Biochemicals). Clones for sequence analysis were obtained by exonuclease III digestion of plasmid DNAs to generate a series of nested deletions (22). Double-stranded template DNA was prepared as suggested by the supplier (U.S. Biochemicals) of the Sequenase sequencing kit and used in reactions with the pUC/M13 17-mer universal forward primer or the reverse sequencing primer (Promega Corp., Madison, Wis.) and α-^{35}S-dATP (3,000 Ci/mmol; New England Nuclear, Boston, Mass.). The complete sequences of both DNA strands were obtained. Nucleotide sequence data were analyzed with the MacVector and AssemblyLign programs from IBI (New Haven, Conn.), with the University of Wisconsin Genetics Computer Group programs on a Vax Workstation, and by BLAST searches at the National Center for Biotechnology Information.

Expression of urease in *E. coli* and *S. gordonii* DL1. To analyze expression of the urease enzyme of *S. salivarius* in recombinant *E. coli* and *S. gordonii*, cells were grown in the media described above but the media were supplemented with $NiCl_2$ at concentrations ranging from 0.0 to 1.0 mM. Cells were grown at 37°C and harvested in the mid-exponential phase of growth. Cells were washed once with 10 mM sodium phosphate buffer (pH 7.0)–1 mM $MgCl_2$ and then resuspended in the same buffer at 1/10 of the original culture volume. Urease activity in intact cells was measured and normalized to cell dry weight. Urease activity measurements following permeabilization of cells with toluene or toluene-acetone mixtures gave the same results as intact cell measurements. Assays were performed in a final volume of 500 μl in 50 mM potassium phosphate buffer (KPB; pH 7.0). Reactions were initiated by addition of urea to a final concen-

tration of 50 mM. Urease activity was quantified by measuring ammonia released from urea by using the Sigma Ammonia Color Reagent with ammonium sulfate as the standard. One unit of urease activity was defined as the amount of enzyme required to hydrolyze 1 μmol of urea per min.

Preparation of crude cell extracts for enzyme analysis. *E. coli* DH10B carrying the cloned urease genes was grown in L broth (2 liters) with chloramphenicol, 70 μM $NiCl_2$, and 0.5 mM phenylmethylsulfonyl fluoride to late exponential phase (optical density at 600 nm ≅ 0.75) and harvested by centrifugation at 8,000 × *g* for 20 min at 4°C. The cells were washed with wash buffer, which contained 10 mM KPB (pH 7.0), 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride, and resuspended in 5 ml of the same buffer. Cells were disrupted by sonication on ice with 20-s, 350-W pulses for 4 min, and the supernatant was recovered after centrifugation at 14,000 × *g* for 20 min at 4°C. Cell extracts of *S. salivarius* 57.I and *S. gordonii* DL1 harboring the plasmid-borne urease genes were prepared from 2-liter static cultures grown in BHI broth supplemented with 20 mM glucose, 20 mM D,L-threonine, and 0.5 mM phenylmethylsulfonyl fluoride to late exponential phase. For *S. gordonii* DL1 harboring the cloned urease genes, 70 μM $NiCl_2$ and 250 μg of spectinomycin per ml were also added to the growth medium. Cells were disrupted by homogenization with a Tissue Tearor homogenizer (Biospec Products, Inc., Bartlesville, Okla.) at maximum speed on ice with one-third of a volume of glass beads (0.1-mm average diameter) for 12 min and centrifuged at 8,000 × *g* for 20 min. The supernatants were then clarified by centrifugation at 14,000 × *g* for 20 min at 4°C.

Purification of urease and protein electrophoresis. Cell extracts (4 ml; prepared as described above) from *E. coli* MC12, which carried the cloned urease genes, were applied to a DEAE Bio-Gel Agarose (Bio-Rad, Richmond, Calif.) column (17 by 3.5 cm) previously equilibrated with wash buffer. The column was washed until the optical density at 280 nm was ≤0.02, and bound protein was eluted by using a linear NaCl gradient (0 to 1.0 M) in wash buffer. Fractions (5 ml) were collected, and enzyme activity in each fraction was measured. Three fractions containing the most activity were pooled, concentrated in a Centricon concentrator with a 30-kDa-pore-size membrane (Amicon Inc. Beverly, Calif.), and applied to a Bio-Gel P-2 (Bio-Rad) gel filtration column for desalting. The urease enzyme was further purified by affinity chromatography on a hydroxyurea column, which was prepared by chemically coupling hydroxyurea to activated CH-Sepharose (Pharmacia Biotech, Inc., Piscataway, N.J.), as previously described (12). The column was equilibrated with wash buffer, protein was applied, and the column was washed until the optical density at 280 nm of the eluent was less than 0.02. Urease activity was recovered by using a linear gradient of 0 to 1.0 M NaCl in wash buffer. All stages of the purification were performed at 4°C. Denaturing and reducing gel electrophoresis was carried out by using the buffers of Laemmli (27), as detailed previously (9), with molecular weight standards from Life Technologies. Protein was detected by silver staining (20).

Biochemical analysis of the *S. salivarius* urease. To determine the optimum pH for activity, protein samples were preincubated for 5 min at 37°C in the presence of 50 mM KPB or 50 mM Tris-HCl, potassium citrate, or citric acid-phosphate buffer over a pH range from 2.5 to 8.0; urea was then added to a final concentration of 50 mM; and urease activity was determined as described above. The pHs of the reaction mixtures were measured at the beginning and end of each experiment. The optimum temperature for urease activity was determined by equilibration of protein samples at various temperatures between 5 and 80°C for 5 min in 50 mM KPB, pH 7.0, and urease activities were determined. Kinetic parameters of the urease preparations were measured by monitoring the rate of urea hydrolysis as a function of urea concentration in 50 mM KPB, pH 7.0. Samples were equilibrated to 37°C for 5 min, and reactions were allowed to proceed in the presence of substrate for 5 min. All reaction conditions and unit definitions were otherwise as described above.

In vitro pH drop experiments. The glycolytic and ureolytic capacities of bacteria were assessed by using a modification of the protocol of Belli and Marquis (4). Cells were grown to mid-exponential phase in BHI broth with 10 μM $NiCl_2$ and 250 μg of spectinomycin per ml. Cells were harvested, washed with 50 mM KCl–1 mM $MgCl_2$, and resuspended in 50 mM KCl–1 mM $MgCl_2$ at 1/10 of the original volume. Samples were equilibrated to room temperature, suspensions were titrated to a pH of 7.2 with 1 N KOH, and pH drops were initiated by addition of 55.6 mM glucose and 0, 2, 5, or 10 mM urea.

Nucleotide sequence accession numbers. The complete sequence of the urease gene cluster reported in this article has been deposited with GenBank and bears accession number U35248. The sequence of the PCR product derived from an internal portion of the *S. salivarius ureC* gene was previously deposited and bears GenBank accession number U22950.

RESULTS

Isolation of the urease gene cluster from *S. salivarius* 57.I. Two genomic libraries were constructed in the intermediate-copy-number plasmid pSU21 (3). These libraries were screened with a 0.6-kbp PCR product internal to the *S. salivarius ureC* gene (10), which we had previously isolated by using degenerate primers specific to conserved amino acid sequences in the UreC proteins of bacteria and the jack bean urease. Two

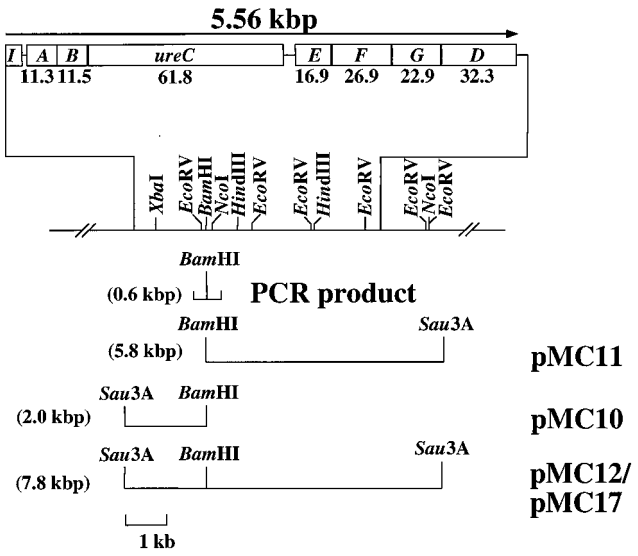

FIG. 1. Schematic diagram of the *ure* gene cluster of *S. salivarius* 57.I showing the gene order and arrangement; the molecular mass in kilodaltons of each gene product; the location of the PCR product used to identify clones; a partial restriction map of the locus and adjacent DNA; and the inserted DNA in plasmids pMC10, pMC11, pMC12, and pMC17.

positive clones, containing a 2.0-kbp DNA fragment (pMC10) and a 5.8-kbp DNA fragment (pMC11) from *S. salivarius* 57.I, were identified (Fig. 1). Nucleotide sequence analysis indicated that the inserted DNAs did not have homology with each other but that the clones had one common point for fusion to the plasmid vector, this being the *Bam*HI site contained within the 0.6-kbp PCR product (Fig. 1). Hybridization of DNA fragments from the two clones at high stringency with total chromosomal DNA of *S. salivarius* 57.I digested with appropriate restriction enzymes further confirmed the source of the DNA and demonstrated that the clones were contiguously arranged on the *S. salivarius* 57.I chromosome (data not shown).

Nucleotide sequence analysis of the urease gene cluster. The complete nucleotide sequences of both strands of the inserted DNA in pMC10 and pMC11 were determined from a series of exonuclease III-generated, nested deletion mutants of both recombinant plasmids (Fig. 1). Translation of the nucleotide sequences revealed the 3′ end of a partial open reading frame (ORF) followed by six complete ORFs that were arranged contiguously on the clones and transcribed in the same direction (data not shown). The six complete ORFs were found to have significant homologies, and to correspond closely in molecular mass, with previously characterized urease structural and accessory genes (Fig. 1). Thus, they have been designated *ureA*, *ureB*, *ureC*, *ureE*, *ureF*, *ureG*, and *ureD*, consistent with existing homologs from most bacterial ureases. A database search (BLAST) with the partial ORF located 3′ to the *ureA* gene indicated that it was homologous to the *ureI* gene of *Helicobacter pylori*, less homologous to two putative ABC transporters (accession numbers, S44146 and Z46523), and only questionably similar to the *ureI* gene of *Bacillus* sp. (29). Thus, the gene encoding this ORF has been designated the *S. salivarius ureI* (Fig. 1). UreI protein homologs are believed to be involved in energized nickel uptake in conjunction with UreH (13, 29).

For brevity, the nucleotide sequence has not been included, but the salient details of the sequence analysis are presented here. The *ure* genes were, on average, 40% G+C, consistent

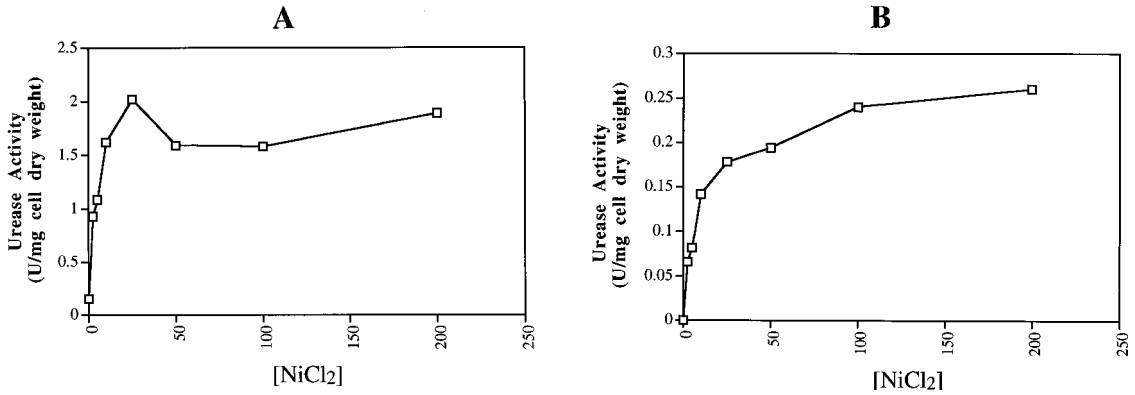

FIG. 2. Effects of supplementation of growth media with nickel chloride on urease activity expressed from plasmid clones in *E. coli* MC12 (A) and *S. gordonii* MC17 (B). Cells were grown and treated and urease specific activity was measured as described in Materials and Methods. No urease activity was detected in control experiments in which the host cells carried only the vector, i.e., *E. coli* DH10B [pSU21] or *S. gordonii* DL278, regardless of the nickel concentration of the growth medium (data not shown). Activity is expressed as units (micromoles of urea hydrolyzed per minute) per milligram of cell dry weight.

with their origin being *S. salivarius* (18). We have assigned a putative promoter immediately 5′ to *ureA* on the basis of proximity, reasonable sequence conservation with existing promoter elements of bacteria, and good evidence of function (see below). The presence of terminators within untranslated portions of the *ure* gene cluster was not detected by use of computer algorithms. Approximately 120 bp from the 3′ end of the *ureD* gene, a sequence with characteristics of a Rho-independent terminator was noted ($\Delta G = -9.2$ kcal [ca. -38 kJ]/mol). It seems unlikely that transcripts arising within the *ure* gene cluster proceed well beyond *ureD*, since homology searches with 1.5 kbp of completely sequenced DNA 3′ to *ureD* did not support the idea that the ORFs located in this region were involved in urea metabolism. Likewise, urease expression in clones lacking the DNA 3′ to *ureD* was not affected (data not shown).

The amino acid sequences of the *S. salivarius* UreA to UreI proteins and the homologies between these proteins and those homologs for which there were sequence data were evaluated. To briefly summarize the salient results of these analyses, the molecular masses of the *S. salivarius* UreA to UreG proteins (Fig. 1) were found to correspond well to those of other bacterial Ure proteins. For the catalytic α subunit, the conserved histidine residues that were shown by site-directed mutagenesis of the *K. aerogenes ureC* gene (38) to be required for nickel binding (His-134, -136, and -246), substrate binding (His-219), and catalysis (His-320), were found to be conserved in relative positions (positions 139, 141, 251, 224, and 325, respectively) in the *S. salivarius* 57.I UreC. The presumed active site in the α subunit of the *S. salivarius* enzyme, MVCHHLD (amino acid positions 326 to 332), did not show any deviation from the consensus urease active site sequence in UreC (31, 48). Other histidine residues in the β (His-39 and -41) and γ (His-97) subunits which have been shown to have functional significance (38) were similarly conserved. The nickel-binding, polyhistidine tail that is in the C terminus of the *K. aerogenes* UreE protein (28, 46) was not present in the deduced amino acid sequence of the *S. salivarius* UreE. Also, the ATP and GTP binding site (P loop; positions 12 to 19) present in the UreG proteins of other bacteria was identified by using the Genetics Computer Group MOTIFS program. Finally, the *S. salivarius* partial UreI ORF is particularly hydrophobic, demonstrating five strongly hydrophobic domains in the sequence thus far obtained (data not shown), and this suggests that it is localized at least partially in the cytoplasmic membrane.

Expression of the *S. salivarius* urease genes in *E. coli* and in *S. gordonii*. To reconstruct a complete urease gene cluster, the 2.0-kbp *Bam*HI fragment was gel purified from pMC10 (Fig. 1). The fragment was then ligated to *Bam*HI-digested, phosphatase-treated pMC11 (Fig. 1). Following transformation, screening by colony hybridization, and analysis of positive clones by restriction digestion and gel electrophoresis, clones with the correct configuration were identified. Nucleotide sequence analysis of the recombinants was done with primers located approximately 100 bp from the 3′ end of the *Bam*HI site in *ureC* to ensure that an intact reading frame existed following ligation at the *Bam*HI site. A single clone, designated pMC12 (Fig. 1), which contained the entire urease gene cluster inserted in the opposite orientation to the *lacZ* promoter and operator in pSU21, was selected for further analysis.

To introduce the intact *ure* cluster into a vector which could replicate in the streptococci, the insert in plasmid pMC12 was subcloned onto an *E. coli*-streptococcal shuttle vector, pDL278 (16), by using *Sst*I and *Sph*I sites in the polylinker of pSU21 and ligated onto *Sst*I-*Sph*I-digested pDL278. *E. coli* was transformed with the ligation mixture, and spectinomycin-resistant clones containing inserted DNA were screened by restriction digestion and gel electrophoresis. The chimeric plasmid, designated pMC17, was determined to have the entire 7.8-kbp insert of pMC12 (Fig. 1) present in the opposite orientation to the *lacZ* promoter in pDL278. This plasmid was used to transform *S. gordonii* DL1 to spectinomycin resistance. Plasmid DNA was isolated from transformants of *S. gordonii* DL1, analyzed by restriction digestion, and found to be indistinguishable from that used for the transformation.

Urease activities of *E. coli* harboring pMC12 and of *S. gordonii* DL1 carrying pMC17 were examined in exponentially growing cells (optical density at 600 nm ≅ 0.6). Since previous reports indicated that it was necessary to supplement the growth media of recombinant *E. coli* strains, carrying a cloned urease, with nickel in order to obtain optimal enzyme activity (26, 29) and because the clones likely did not contain an intact nickel transport system, the effects of supplementation of the medium with nickel were concurrently examined. In the absence of added nickel, urease was detected in *E. coli*, although the level of expression was quite low (Fig. 2A). With no nickel supplementation, urease activity was not detected in *S. gordonii* (Fig. 2B). Supplementation of media with as little as 2.5 μM $NiCl_2$ resulted in markedly enhanced urease activity in both organisms (Fig. 2). For *E. coli* MC12, optimal activity could be

TABLE 2. Purification of the urease enzyme from *E. coli* MC12[a]

Purification step	Total activity (U)[b]	Total protein (mg)	% Recovery	Sp act (U/mg of protein)[c]	Purification (fold)[d]
Cell extract	1,480	168.0	100	8.8	1.0
DEAE Bio-Gel Agarose	639	39.4	43	90.0	10.2
Bio-Gel P-2	330	4.3	22.3	137.8	15.7
Hydroxyurea affinity	319	3.3	21.6	1,700.0	193.2

[a] Protein was purified from cell extracts of *E. coli* MC12 grown and prepared as detailed in Materials and Methods.
[b] Total activity represents urease activity recovered in all fractions at each step.
[c] Specific activity represents the activity in that fraction which had the peak activity. A subset of fractions with the highest specific activity were pooled and used to further purify the enzyme or, for the final step, to determine the K_m and optimal reaction conditions.
[d] Fold purification was calculated on the basis of those fractions which were selected to calculate specific activities.

observed when the medium was supplemented with approximately 25 μM $NiCl_2$ and inhibition of bacterial growth became apparent at $NiCl_2$ concentrations above 70 μM. In *S. gordonii* MC17, urease activity increased with added $NiCl_2$ up to 200 μM (Fig. 2B), but as with *E. coli*, significant growth inhibition was observed when the concentration of added nickel exceeded 70 μM. The concentration of nickel required for optimal urease activity in *E. coli* MC12 was higher (about 70 μM) in L broth, and the MIC of nickel was higher (>1 mM), presumably because of chelation of this toxic metal by medium components (data not shown).

Biochemical characterization of the urease of *S. salivarius* 57.I. The urease enzyme was purified from cell extracts of *E. coli* MC12 as summarized in Materials and Methods. This purification protocol yielded a urease enzyme preparation with a specific activity of 1,700 U/mg of protein (Table 2). The purified enzyme was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and silver staining. Although the preparation was not completely homogeneous, as judged after two cycles of silver staining of 2 μg of the purified material, there was a major enrichment of a protein with an estimated molecular mass of 62 kDa (Fig. 3), close to the estimated molecular mass of the large subunit of the enzyme (61.8 kDa). Two stained species of <15 kDa, which may represent the β and γ subunits, were also substantially enriched in the purification. The levels of purification and specific activity of the protein preparation derived by using the protocol mentioned above were well within the ranges reported for numerous other ureases which have been biochemically characterized (33). Enzyme thus purified was used for measurement of biochemical parameters.

It was found that urease activity in cell extracts prepared from the streptococcal hosts was retained for several days at 4°C. However, all attempts (>20) to purify the urease enzyme from the parent organism or from *S. gordonii* MC17 failed, despite the use of various permutations and modifications of the above-described steps in conjunction with combinations of protease inhibitors, because activity was not retained throughout the purification procedures. For this reason, the properties of the enzyme purified from *E. coli* were compared with those of the enzyme present in cell extracts prepared from *S. gordonii* MC17 and from *S. salivarius.*

Biochemical characterization of the *S. salivarius* urease. Urease activity was optimal at about pH 7.0, although the enzyme was similarly active over a pH range from 6.0 to 7.0 (Fig. 4). Greater than 65% of the activity measurable at pH 7.0

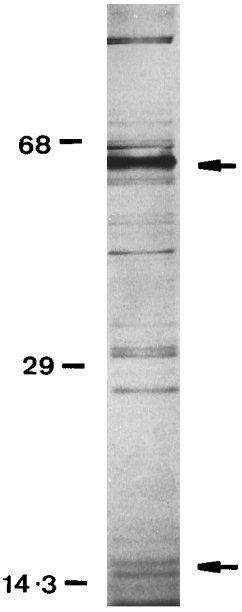

FIG. 3. SDS-PAGE and silver staining of purified recombinant urease. Urease expressed in *E. coli* MC12 was purified and subjected to SDS-PAGE as described in the text. Two micrograms of the purified material was separated by electrophoresis in a 15% polyacrylamide gel and silver stained. The arrows indicate the positions of stained species which may represent the UreC protein (62 kDa) and the two small subunits (UreAB) of the active form of the *S. salivarius* urease.

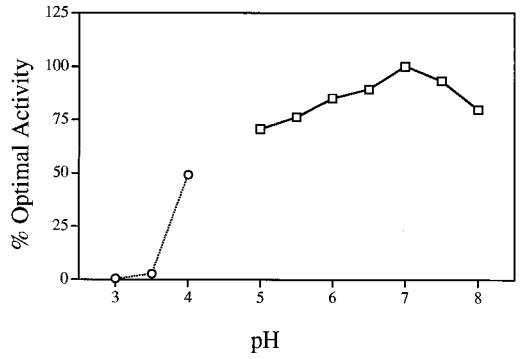

FIG. 4. Urease activity as a function of pH. Purified enzyme from *E. coli* MC12 was tested for optimal activity in 50 mM KPB (squares) at the pH values indicated as detailed in Materials and Methods. Samples were equilibrated at the desired pH at 37°C for 5 min. Reactions were initiated by addition of urea to a final concentration of 50 mM. After 5 min, reactions were terminated and ammonia was measured as detailed in the text. Before and after each reaction, the pH of the reaction mixture was measured. No change in pH was observed in any case. Essentially the same results were obtained with Tris-HCl and potassium citrate buffers. The relative activities at low pH of the various enzyme preparations as determined in 50 mM citrate-phosphate buffer to demonstrate the rapid decline in activity observed at pH values below 4.0 are also shown (circles).

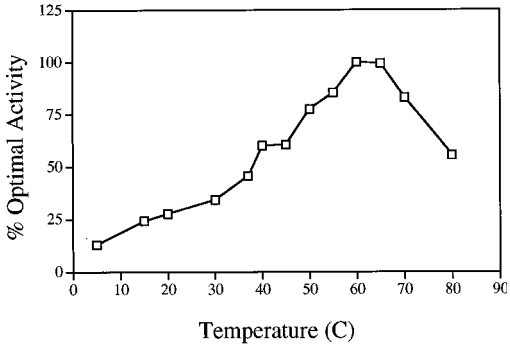

FIG. 5. Urease activity as a function of reaction mixture incubation temperature. Samples were equilibrated at the desired temperature for 5 min in KPB (50 mM; pH 7.0). Reactions were initiated by the addition of 50 mM urea, and ammonia released was measured as described in the text.

was retained at pH values as low as 5.0, but activity decreased sharply below pH 3.5. The temperature optimum for activity was 60°C, with 55% of the optimum activity remaining at 80°C and a rapid decline in activity occurring at temperatures lower than 30°C (Fig. 5). The K_m of the purified recombinant urease was calculated to be about 3.7 mM. The properties of the purified recombinant enzyme were compared with those of the urease in cell extracts from *S. salivarius* 57.I and *S. gordonii* MC17 (Table 3). In all cases, the purified *E. coli* preparation and the enzyme produced in the oral streptococci behaved almost identically. The only minor difference noted was that the pH optima of the crude preparations were somewhat broader than that of the purified enzyme. This was likely due to macromolecular crowding or local buffering effects, since crude *E. coli* MC12 extracts displayed a similarly broad urease pH optimum (data not shown).

Modulation of glycolytic acidification by recombinant ureolytic plaque streptococci. Dental caries occurs as a result of dissolution of the tooth enamel through glycolytic acidification by plaque bacteria. Caries is largely a disease arising from a shift in dental plaque microbial composition away from a healthy, or basic, microflora to a cariogenic microflora (7). A cariogenic microflora consists predominantly of aciduric and highly acidogenic species, such as *Streptococcus mutans* and *Lactobacillus casei*, which do not produce ammonia. Our hypothesis is that ammonia-producing recombinant bacteria may reduce the cariogenic potential of dental plaque by maintaining a relatively alkaline environment, thus preventing significant enamel dissolution and suppressing the emergence of highly aciduric bacteria (8), such as *S. mutans*. To begin to determine if such a strategy was feasible, the potential for base production to modify the extent of environmental acidification as a result of glycolysis was assessed by in vitro pH drop experiments (Fig. 6). Cell suspensions of the dental plaque microorganism *S. gordonii* DL1, harboring the recombinant plasmid pMC17 or the control plasmid pDL278, lowered the pH rapidly to values near 4, from an initial value of 7.2, after addition of 55.6 mM glucose (Fig. 6). When as little as 2 mM urea was added to the cell suspensions simultaneously with glucose, the ureolytic recombinant *S. gordonii* was able to dramatically blunt glycolytic acidification (Fig. 6A). Inclusion of 5 mM urea resulted in a pH rise, while 10 mM urea yielded a sustained pH rise to approximately 8.7. Addition of urea, but not glucose, to cells (data not shown) resulted in a rapid alkalization of the suspensions to near pH 9.0, close to the pK_a of ammonium ions ($\cong$ 9.2). *S. gordonii* DL1 carrying only the vector plasmid, pDL278, had no capacity to modulate glycolytic acidification when provided with urea (Fig. 6B). When just urea was added to *S. gordonii*(pDL278) suspensions, no rise in pH was seen (data not shown). Rather, a very slow decline in pH, probably due to metabolism of endogenous stores of carbohydrate, was noted.

TABLE 3. Summary of biochemical characteristics of the ureases produced by *E. coli* MC12, *S. salivarius* 57.I, and *S. gordonii* MC17[a]

Source of enzyme[b]	Optimum pH	Optimum temp (°C)	K_m (mM)
E. coli MC12	7.0	60	3.70 ± 1.40
S. salivarius 57.I	6.5–7.0	60	3.84 ± 2.12
S. gordonii MC17	6.0–7.0	60	3.70 ± 0.99

[a] The values expressed were derived from a minimum of three experiments, each performed in triplicate.

[b] The protein preparations utilized to determine pH and temperature optima and K_m values were the purified protein (1,700 U/mg of protein) from *E. coli* MC12 and cell extracts from *S. salivarius* 57.I (1.80 U/mg of protein) and *S. gordonii* MC17 (1.25 U/mg of protein).

DISCUSSION

Substantial evidence supports the theory that ureolysis in the human oral cavity is a critical factor in microbial ecology and in oral health and diseases. Yet virtually no work has been done on molecular aspects of ureases of oral bacteria commonly isolable from the tissues of the mouth. This report provides the first description of the genes and enzyme characteristics of the urease of a human oral isolate, *S. salivarius*, which is present in abundance on the soft tissues of the mouth although seldom found in dental plaque.

The organization of the urease genes was similar to that of other urease operons, although some minor differences in spacing and gene order were evident. It appears from the fact that the enzyme is expressed at high levels in *S. gordonii* that a functional promoter is located immediately upstream of *ureA*. Further analysis of the transcriptional organization and regulation of the *S. salivarius* urease genes will be needed to assess the functionality of other potential regulatory elements.

The urease enzyme has biochemical characteristics in common with a variety of other urease enzymes. With regard to oral ureolysis, though, the enzyme has several characteristics which are germane to function in the human oral cavity. First, the K_m is such that the enzyme should function well with concentrations of urea normally found in the mouth (3 to 10 mM). Likewise, the pH optimum of the enzyme is compatible with its functioning at high levels in vivo in oral streptococci. Specifically, since the cytoplasmic pH of oral streptococci appears to be maintained at 0.5 to 1 pH unit higher than the environmental pH, the enzyme should function at nearly half-maximal activity even at values which are considered extreme for dental plaque (pH 4.0). Although not completely surprising (34), the optimum temperature for the *S. salivarius* urease was high, i.e., 60°C. By biochemical and microbiologic analyses, we have confirmed that strain 57.I is a true *S. salivarius* strain and not a strain of *Streptococcus thermophilus*, which is a closely related, thermotolerant, ureolytic species. It will be of interest to contrast the urease genes of these two species from an evolutionary standpoint, and analysis of the urease primary sequences of these organisms may provide insight into the basis for thermotolerance of the enzymes.

Our principal interests in the ureases of oral bacteria stem from the mostly indirect evidence that these enzymes are key in various aspects of oral health and diseases. In addition to

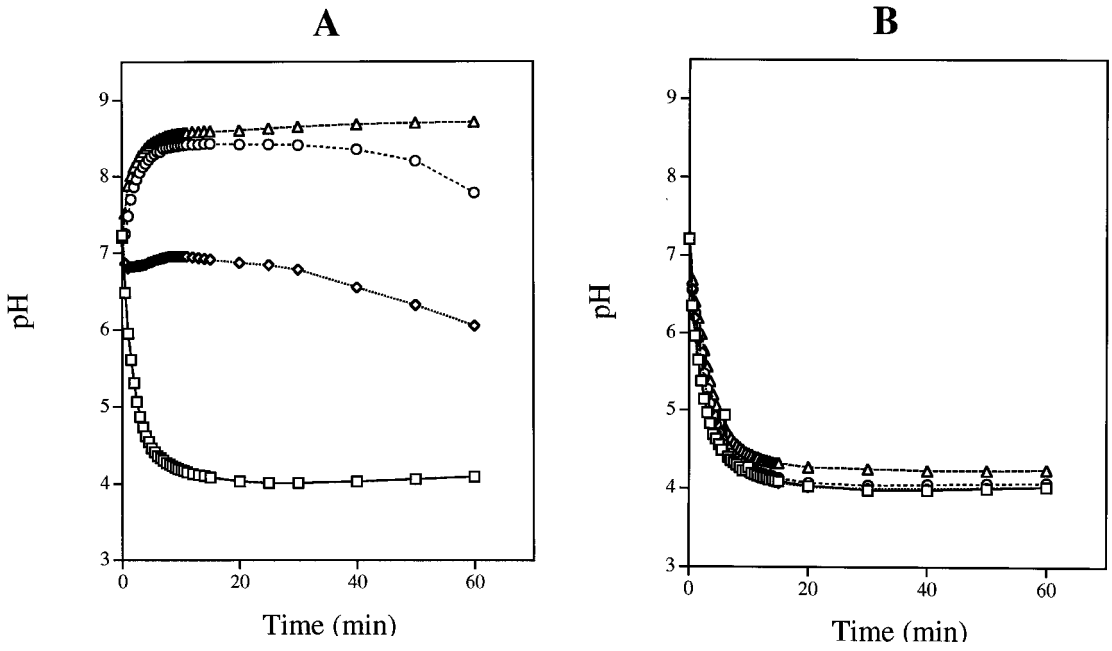

FIG. 6. Effects of urea and glucose on environmental acidification by *S. gordonii* DL1 carrying the urease genes (MC17) (A) or the plasmid vector alone (DL278) (B). Cell suspensions were titrated to pH 7.2 with KOH, and glycolysis was initiated by the addition of 55.6 mM glucose in the presence of 0 (squares), 2 (diamonds), 5 (circles), or 10 (triangles) mM urea. The results presented here represent those obtained on separate occasions ($n = 4$ for MC17; $n = 3$ for DL278) when cells were grown, cell suspensions were prepared, and pH drop experiments were performed.

providing fundamental information about the biochemistry and genetics of urease of oral bacteria, this study begins to lay the groundwork for a more detailed study of the role of ureases in microbial ecology, bacterial physiology, dental plaque pH homeostasis, and oral diseases. In this study, we have demonstrated that normally nonureolytic strains of dental plaque bacteria can be genetically engineered to produce urease at high levels. Most importantly, our data indicated that physiologically relevant levels of urea (2 to 10 mM) can dramatically alter environmental acidification even in the presence of a significant glucose excess. The ability to construct various strains of plaque bacteria expressing urease will allow evaluation of the role of ureases in oral disease in a manner that has previously been impossible. For example, strains of urease-producing *S. gordonii* or *S. mutans* could be used to test whether such strains can modulate the cariogenic potential of dental plaque when implanted in animal models. Such strains may be extremely effective at controlling caries, since in addition to neutralizing acids in plaque, the elevated pH may also prevent a shift in plaque ecology toward a more cariogenic microflora. Reasonably good animal models for examination of calculus formation and periodontal diseases also exist (36). The ability to use genetically engineered, base-producing oral bacteria in controlled experiments should provide valuable information which may allow the enhancement of the base producing capacities of human dental plaque or the rational design of therapeutic agents which exploit urea or ureases. The knowledge gained from such studies may also lead to the development of strains that could eventually prove useful for control of dental caries by replacement therapy.

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