INFECTION AND IMMUNITY, Feb. 1999, p. 504–512 Vol. 67, No. 2
0019-9567/99/$04.00+0

Genetic and Physiologic Characterization of Urease of *Actinomyces naeslundii*

EVANGELIA MOROU-BERMUDEZ AND ROBERT A. BURNE*

Center for Oral Biology and Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York 14642

Received 27 August 1998/Returned for modification 7 October 1998/Accepted 4 November 1998

Ammonia production from urea by ureolytic oral bacteria is believed to have a significant impact on oral health and the ecological balance of oral microbial populations. In this study we cloned and characterized the urease gene cluster of *Actinomyces naeslundii*, which is one of the pioneer organisms in the oral cavity and a significant constituent of supragingival and subgingival dental plaque in children and adults. An internal fragment of the *ureC* gene of *A. naeslundii* WVU45 was initially amplified by PCR with degenerate primers derived from conserved amino acid sequences of the large catalytic subunit of urease in bacteria and plants. The PCR product was then used as a probe to identify recombinant bacteriophages carrying the *A. naeslundii* urease gene cluster and roughly 30 kbp of flanking DNA. Nucleotide sequence analysis demonstrated that the gene cluster was comprised of seven contiguously arranged open reading frames with significant homologies at the protein and nucleotide sequence levels to the *ureABCEFGD* genes from other organisms. By using primer extension, a putative transcription initiation site was mapped at 66 bases 5′ to the start codon of *ureA*. A urease-deficient strain was constructed by insertion of a kanamycin resistance determinant within the *ureC* gene via allelic replacement. In contrast to the wild-type organism, the isogenic mutant was unable to grow in a semidefined medium supplemented with urea as the nitrogen source and was not protected by the addition of urea against killing in moderately acidic environments. These data indicated that urea can be effectively utilized as a nitrogen source by *A. naeslundii* via a urease-dependent pathway and that ureolysis can protect *A. naeslundii* against environmental acidification at physiologically relevant pH values. Therefore, urease could confer to *A. naeslundii* critical selective advantages over nonureolytic organisms in dental plaque, constituting an important determinant of plaque ecology.

Ureases are nickel-containing, multisubunit enzymes that catalyze the hydrolysis of urea to ammonia and carbon dioxide, with a net increase in environmental pH. They are highly conserved proteins found in a number of plants, bacteria, fungi, and algae (45). In prokaryotes, urea hydrolysis can confer protection against killing in acidic environments (59) or can provide ammonia, which is a preferred nitrogen source for many bacteria (17). There is also evidence that some organisms, such as *Ureaplasma ureolyticum* (62) and some alkalophiles (32), may use ureolysis to generate a proton motive force that can drive ATP synthesis.

Expression of a catalytically active urease in bacteria is usually directed by at least seven genes, which in general are arranged in operons (16, 46). Three of the genes encode the structural subunits of urease: the large catalytic subunit (α), encoded by the *ureC* gene, and the two smaller subunits (β and γ), which are the products of *ureB* and *ureA*, respectively. The three subunits can associate in an $(\alpha\beta\gamma)_3$ stoichiometry to form the urease apoenzyme (31). Activation of the apoenzyme involves the incorporation of six nickel ions per active trimeric molecule and is accomplished by the coordinated action of four accessory proteins, encoded by *ureD*, *-E*, *-F*, and *-G* (39, 48). The roles of each of these proteins in the urease holoenzyme assembly process are beginning to be elucidated. A current model for urease apoenzyme activation (52) proposes that UreD functions as a molecular chaperone which maintains the apoenzyme in a competent state, able to receive nickel ions from the nickel donor, UreE. In this model, UreG, which has an identifiable ATP- and GTP-binding domain, participates in some energy-dependent stage of urease activation (47, 67). Additional urease-associated genes, involved in either the regulation of urease expression (*ureR*) (21, 50) or the transport of nickel into the cell (*nixA* and *ureH*) (37, 44), have also been described for ureolytic organisms.

Bacterial urease activity can contribute to the development of several pathologic conditions in humans, such as gastritis (9), pyelonephritis (49), and urinary tract stone formation (42). Substantial amounts of urea are also present in human oral secretions, such as saliva and gingival crevicular fluid (28), and a number of indirect observations have suggested an involvement of ureolysis in the pathogenesis of oral diseases (3, 25, 28, 43, 54, 70). Ureolytic activity in supragingival dental plaque can counteract the effects of microbial glycolytic activity and thus prevent plaque acidification (15, 34, 58). By helping to maintain plaque pH at neutral levels, ureolysis could inhibit demineralization of dental enamel, which otherwise occurs in acidic environments (23), and may also prevent ecological shifts in dental plaque commonly associated with caries development (10). For these reasons, alkali generation via ureolysis in the oral cavity can potentially be an important inhibitor of dental caries formation (34, 54). Conversely, the production of ammonia by ureolytic organisms in subgingival plaque may have detrimental effects on periodontal tissues. These include promotion of the precipitation of normally soluble ions from saliva and gingival crevicular fluid, which is induced by alkaline pH and which can lead to the formation of subgingival calculus (25, 38), as well as possible contributions to inflammatory processes that lead to periodontal disease (3, 28, 29).

* Corresponding author. Mailing address: Center for Oral Biology and Department of Microbiology and Immunology, University of Rochester Medical Center (Box 611), 601 Elmwood Ave., Rochester, NY 14642. Phone: (716) 275-0381. Fax: (716) 473-2679. E-mail: robert_burne@urmc.rochester.edu.

Dental plaque isolated from healthy tissues demonstrates significant ureolytic activity (60), but the organisms that are responsible for this activity and the extent to which particular species contribute to total plaque ureolysis have not been identified (26, 61). Strains of *Actinomyces naeslundii* genospecies 1 (33) are gram-positive, facultatively anaerobic bacteria, characteristically rich in G+C DNA content, and are usually urease positive upon isolation (55, 74), in contrast to *Actinomyces viscosus* (*A. naeslundii* genospecies 2), which is generally urease negative. Strains of *A. naeslundii* are of special interest because they are found almost uniquely in the mouth, they are early colonizers of the oral cavity (24, 64), and they comprise significant portions of both supragingival and subgingival dental plaque (5, 73). *A. naeslundii* has been implicated in the pathogenesis of root caries (57, 69) and periodontal diseases (27, 63), although these associations have never been unequivocally established (6). The organism does not appear to be involved in the development of coronal caries (73), and in fact it is most often isolated from sites with low cariogenic activity.

Our working hypothesis is that the ability of *A. naeslundii* to colonize the oral cavity before the emergence of acidogenic organisms and to generate ammonia from urea in both supragingival and subgingival plaque may have a significant impact on the ecological balance in oral biofilms. To begin to understand the role of ureolysis by *A. naeslundii* in the physiology of this organism, and eventually in oral ecology and disease development, we isolated and characterized the urease gene cluster from this organism and constructed a urease-deficient, otherwise isogenic mutant strain. The isogenic mutant was compared to the wild-type organism in a number of in vitro experiments to determine the physiologic significance of urease in *A. naeslundii*.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and reagents. *A. naeslundii* WVU45 (ATCC 12104) (14) and ANUC1 (this study) were grown in either brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) or *Lactobacillus*-carrying medium (22). For comparisons of the growth of *A. naeslundii* on different nitrogen sources, a semidefined medium (*Actinomyces* defined medium) (ADM) (7) was used with the modifications specified in Results. *Escherichia coli* KW251 and DH10B were grown in Luria broth with aeration, and phages were propagated as described by Sambrook et al. (56). The antibiotics used in this study were ampicillin (100 μg/ml), kanamycin (50 μg/ml), and streptomycin (50 μg/ml). All chemical reagents and antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.).

DNA manipulations. Chromosomal DNA from *Actinomyces* was isolated as described by Donkersloot et al. (20). Plasmid DNA was extracted from *E. coli* by the rapid-boiling method (56). DNA to be used for subcloning or nucleotide sequence analysis was extracted from *E. coli* by using the QIA Prep Spin Plasmid Kit (Qiagen, Inc., Chatsworth, Calif.) or isolated by the method of Birnboim and Doly (2) and was further purified by centrifugation to equilibrium in cesium chloride-ethidium bromide (56). DNA was extracted from recombinant λ phages by the method of Chisholm (13). Restriction and DNA-modifying enzymes were obtained from Life Technologies Inc. (LTI) (Bethesda, Md.), or from U.S. Biochemicals (Cleveland, Ohio).

For the amplification of internal sequences of *ureC* from *A. naeslundii* by PCR, 0.5 μg of chromosomal DNA was mixed with 100 pmol of each primer (Urea-1 [5′-AARATHCAYGARGAYTGGGG-3′] and Urea-3 [5′-GCNGGRTTDAT NGTRTAYTT-3′]). The reaction mixture (100-μl final volume) contained 1.5 mM $MgCl_2$, 75 μM each deoxynucleotide, 1 U of Perfect Match Enhancer (Stratagene, La Jolla, Calif.), and 2.5 U of *Taq* DNA polymerase (LTI). The conditions used for the amplification were as follows: denaturation at 94°C for 1 min, primer annealing at 42°C for 2 min, and extension at 55°C for 2 min for five cycles. Then, for 30 additional cycles, the annealing temperature was increased to 55°C for 2 min and the extension reaction temperature was increased to 72°C for 2 min. PCR products were analyzed by electrophoresis in 1.2% Tris-borate-EDTA agarose gels, and those of the correct estimated size were cloned into the pCRII vector (Invitrogen, San Diego, Calif.).

Southern blotting experiments were carried out by the method of Southern (65) as described by Sambrook et al. (56). DNA probes were labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, Mass.) by using the Random Primers Kit from LTI. Hybridizations were performed at 65°C, and subsequently the blots were washed once in 2× SSC(1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.5% sodium dodecyl sulfate (SDS) and once in 2× SSC plus 0.1% SDS at room temperature, followed by five washes in 0.1× SSC plus 0.5% SDS at 65°C.

An *A. naeslundii* WVU45 genomic library was constructed in bacteriophage λGEM-12 (Promega Corp., Madison, Wis.) according to the instructions of the supplier. Briefly, genomic DNA from *A. naeslundii* was partially digested with *Sau*3AI under conditions that enriched for 15- to 20-kbp fragments. The ends of the fragments were partially filled in by using dATP, dGTP, and Klenow fragment and then ligated to the phage arms, which are the products of an *Xho*I partial digestion of the phage, followed by a partial fill-in reaction with dTTP and dCTP and dephosphorylation. The ligation mixture was then packaged into phage particles (Promega), and the packaged phages were used to infect *E. coli* KW251. For the screening of the genomic library, a total of approximately 3×10^4 plaques were lifted onto nitrocellulose filters (HATF; Millipore Inc., Bedford, Mass.) in duplicate and probed with an [α-^{32}P]dCTP labeled, 587-bp PCR product (3×10^5 cpm/ml of hybridization solution). The PCR product had been purified from a Tris-acetate-EDTA agarose gel by using the Elu-Quik DNA Purification Kit (Schleicher & Schuell, Keene, N.H.). Plaque lifting and hybridization procedures were carried out as described by Sambrook et al. (56) with the hybridization and washing conditions described above.

Nucleotide sequence analysis was performed by the Ladderman dideoxy sequencing method (Takara Shuzo Co., Otsu, Japan). A series of nested deletions were obtained by exonuclease III digestion of plasmid DNA (30), and the generated clones were sequenced by using the pUC/M13 17-mer universal forward primer (Promega). Other primers used were oligonucleotides (18 to 22 bases) complementary to the derived sequences of the *A. naeslundii* urease locus (obtained from LTI) and the reverse sequencing primer (Promega). Sequencing reaction products were labeled with α-^{35}S-dATP (New England Nuclear).

Genetic transfer of exogenous DNA into *A. naeslundii* was performed as described by Yeung and Kozelsky (79) by using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) connected to a pulse controller.

Primer extension analysis. Total RNA was extracted from *A. naeslundii* WVU45 growing exponentially in BHI broth by a protocol described by Yeung (76). Primer extension was performed with the 20-mer 5′-GCGGCGACGACG ATGAGTAG-3′. The protocol used was the one of McKnight and Kingsbury (41), as described by Ausubel et al. (1) with the following modifications: primer annealing was carried out in a buffer containing 1.5 M KCl, 0.1 M Tris (pH 8.0), and 10 mM EDTA at 37°C, and reverse transcription was performed at 42°C.

Urease enzyme assays. To measure urease activity, cells were collected by centrifugation at 2,900 × *g* and washed once in 10 mM sodium phosphate buffer, pH 7.0. The cells were then resuspended in 1 mM sodium phosphate buffer (pH 7.0) and incubated at 37°C in a reaction mixture containing 50 mM potassium phosphate buffer (pH 6.0) and 50 mM urea. The amount of ammonia released was quantitated with the Sigma Ammonia Color Reagent with ammonium sulfate as the standard. Urease specific activity was expressed as micromoles of ammonia produced per minute per milligram of cells (dry weight). Rapid screening for urease activity in recombinant *Actinomyces* strains was performed by streaking onto Bacto urea agar base plates supplemented with 1.5% Bacto Todd-Hewitt broth (Difco) and antibiotics.

Acid-killing experiments. In order to determine whether ureolysis could protect *A. naeslundii* against killing in acidic environments, the wild-type (WVU45) and mutant (ANUC1) strains were grown overnight in ADM containing 0.05% Casamino Acids. The cells were washed once in 10 mM sodium phosphate buffer (pH 7.0) and resuspended in 1/10 of the original volume in citrate-phosphate buffer of pH 7.0, 4.0, or 3.0 with or without the addition of 25 mM urea. The citrate-phosphate buffers had been diluted appropriately so that the phosphate concentration was 10 mM at all pH values. The cell suspensions were incubated at 37°C for up to 6 h. At various time points during that period, 10-μl aliquots were removed from the cell suspensions, serially diluted, plated on BHI plates (plus kanamycin for strain ANUC1), and incubated anaerobically for 3 to 5 days before colonies were counted. The cell viability at each time point was expressed as the percentage of the viable cells (CFU/milliliter of culture) at time zero.

Nucleotide sequence accession numbers. The complete nucleotide sequence of the urease genes from *A. naeslundii* has been deposited with GenBank and bears accession no. AF056321. The individual open reading frames (ORFs) bear accession no. AF048778 through AF048784.

RESULTS

Isolation of the urease gene cluster from *A. naeslundii* WVU45. An approximately 0.6-kbp product was amplified from the *A. naeslundii* WVU45 chromosome by PCR with the degenerate primers Urea-1 and Urea-3. These primers were designed based on conserved amino acid sequences of the α subunits of ureases from a number of organisms (12). The product was cloned into the PCRII vector (Invitrogen) and subjected to nucleotide sequence analysis, which revealed high levels of similarity and identity at the deduced amino acid sequence level to known ureases. Southern blot hybridization

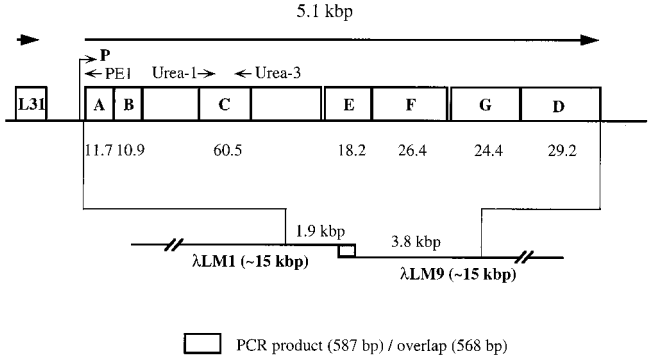

FIG. 1. Schematic representation of the urease gene cluster of *A. naeslundii* WVU45. The arrangement of the genes (top), their locations on the phage clones λLM1 and λLM9 (bottom), and the predicted molecular masses (in kilodaltons) of the corresponding proteins (numbers below the boxes) are shown. The internal fragment that was originally amplified from the chromosome by PCR and used to identify the clones is shown as a shaded box. The relative locations of the primers PE1, Urea-1, and Urea-3 (see text), as well as a putative proximal promoter (P), are also indicated.

of the PCR product, as well as of an internal fragment of the type 2 fimbrial subunit gene of *A. naeslundii* (78) to *A. naeslundii* WVU45 chromosomal DNA under high stringency (data not shown) confirmed that the origin of the product was the *A. naeslundii* WVU45 chromosome.

A genomic library of *A. naeslundii* WVU45 was constructed in phage λGEM-12 as described in Materials and Methods. After screening and three rounds of plaque purification, two phage clones (λLM1 and λLM9) that hybridized to the PCR product under stringent conditions were isolated. Restriction enzyme analysis revealed that the two clones contained approximately 15-kbp inserts and overlapped only at an approximately 0.6-kbp region (Fig. 1). A number of DNA fragments from the two phage clones were subcloned into the plasmid vector pBluescript II (Stratagene) or pGEM7zf(+) or pGEM5zf(+) (Promega), generating various subclones for sequencing.

Nucleotide sequence analysis of the urease gene cluster. Nucleotide sequence analysis of the cloned DNA fragments revealed seven contiguous ORFs, which were transcribed in the same direction (Fig. 1). The first three ORFs were highly homologous, at the nucleotide and deduced amino acid sequence levels, to the genes encoding the three structural subunits of urease in other bacteria: *ureA* (γ), *ureB* (β), and *ureC* (α). The homologies were higher with ureases of gram-positive organisms (up to 74% identity and 87% similarity to *Bacillus* spp.) and were lower, but still significant, with ureases of gram-negative bacteria (up to 64% identity and 78% similarity to *Klebsiella aerogenes*) and plants (58% identity and 73% similarity to the jack bean urease). The remaining four ORFs were somewhat less, but still significantly homologous to the *ureE*, *ureF*, *ureG*, and *ureD* genes, which encode urease accessory proteins involved in the incorporation of nickel into the urease apoenzyme. Of the *A. naeslundii* urease accessory proteins, UreG appeared to be the most highly conserved, with up to 70% identity (84% similarity) to the UreG from *Bacillus* spp., whereas UreD was the least conserved overall (up to 36% identity and 60% similarity to the *Bacillus* UreD). No other ORFs with sequence homology to urease-associated proteins have been identified within 1 kbp of the 5′ or 0.3 kbp of the 3′ region of the urease cluster. A 252-bp ORF with a high degree of similarity at the amino acid level to the L31 ribosomal protein of *Bacillus subtilis* was identified 750 bp 5′ to the coding sequence for *ureA* and was transcribed in the same direction. The 500-bp region between the end of this ORF and the beginning of *ureA* was characterized by a large number of sequences with the potential to form strong stem-loop structures, and it contained two additional putative ORFs transcribed in the same orientation as the urease genes. The deduced amino acid sequences of these two ORFs are 40 and 58 amino acids, respectively, and they do not have homology with known proteins.

The seven ORFs encompassed a region of 5.1 kbp. The G+C content of this DNA averaged 68%, which is typical in the genus *Actinomyces*. A sequence with characteristics of a Rho-independent terminator immediately 3′ to the stop codon for the *ureD* gene was identified by use of computer algorithms. By the same method, no potential terminators appeared to be present within the intergenic regions of the urease gene cluster.

The UreA to -G proteins from *A. naeslundii* appeared to share many of the characteristics common to known bacterial urease proteins. Their calculated molecular masses (Fig. 1) corresponded well to those of the proteins from other bacteria. Amino acid residues in the structural subunits which have been shown to have functional significance in other bacterial ureases were conserved in relative position in the *A. naeslundii* urease. Specifically, histidine residues His-134, His-136, and His-246 of the α subunit of the *K. aerogenes* urease have been shown by site-directed mutagenesis to be involved in nickel binding (53). Histidines were present at positions 137, 139, and 249 of the α subunit of *A. naeslundii* urease. His-219 and His-320 of the *K. aerogenes* α subunit are required for substrate binding and catalysis, respectively (53), and histidine residues were present at positions 222 and 315 of the *A. naeslundii* urease α-subunit. Additional amino acid residues with defined functional significance in the urease enzyme subunits include His-39 and -41 of the β subunit and His-97 of the γ subunit (53), and those were also conserved in the appropriate positions in *A. naeslundii* UreB and UreA, respectively. An amino acid sequence (MVC HHLN) which deviated by only one residue from the consensus for urease active sites (MVCHHLD) (40, 71) was identified at amino acid positions 320 to 326 of the *A. naeslundii* UreC. The conserved ATP- and GTP-binding motif found in other UreG proteins (67) was also identified by the Genetics Computer Group MOTIFS program in *A. naeslundii* UreG (amino acid positions 32 to 39). The deduced amino acid sequence for the *A. naeslundii ureE* gene lacks a polyhistidine tail, which is thought to be involved in nickel binding by UreE of *K. aerogenes* (36, 66).

Mapping of the transcription initiation site by primer extension. Primer extension analysis was performed with a primer (PE1) complementary to the nucleotide sequence between positions 31 and 50 of the *ureA* coding sequence. A single transcription initiation site was identified, corresponding to a cytosine residue located at 66 bases 5′ to the start codon for *ureA* (Fig. 2). At position −4, relative to this transcription initiation site, we identified a hexamer (−TATAAG−) with homology to the *E. coli* σ^{70} promoter −10 consensus sequence (TATAAT), while 19 bases 5′ to this region, a sequence (−TTCACG−) with significant homology to the *E. coli* σ^{70} promoter −35 consensus sequence (TTGACA) is present. The actual −10 region of the *A. naeslundii* urease promoter (−TTGCC−) resembles the −12 consensus sequence of the *E. coli* σ^{54} promoter (TTGC), but no homology to this class of promoters was evident in the respective −24 region. A notable characteristic of the *A. naeslundii* urease promoter region was the unusually high frequency of adenine and thymine phosphonucleotide residues (about 45% within the −100 region).

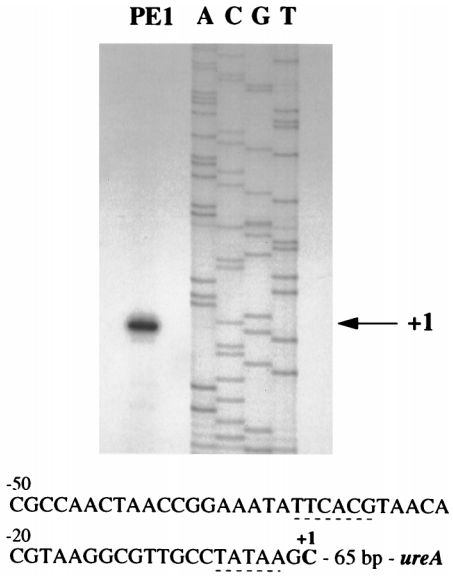

FIG. 2. Mapping of the transcription initiation site by primer extension. Lane PE1, primer extension product obtained with the primer PE1 (see text). The sequencing reactions in lanes A, C, G, and T were obtained with the same primer. Note that the sequencing reactions have been mirror-imaged in order to represent the sequence of the sense DNA strand. The nucleotide sequence of the −50 region relative to the mapped transcription initiation site is presented at the bottom. Two sequences with high homology to canonical −10 and −35 promoter sequences are underlined by dashed lines.

Construction of a urease-deficient *A. naeslundii* strain. The strategy that was followed for the generation of an otherwise isogenic urease-deficient strain is shown in Fig. 3A. A 1.8-kbp *Bam*HI fragment (the second *Bam*HI site was vector derived) containing the 3′ region of *ureA*, *ureB*, and the 5′ region of *ureC* was cloned into the *E. coli* vector pUC19. A 1.3-kbp *Xho*I-*Dra*I fragment from the broad-host-range plasmid vector pJRD215 (19), containing a kanamycin resistance gene that can be expressed in *Actinomyces* spp. (77, 79), was blunt ended and cloned into a unique *Sfi*I site within the *ureC* gene. The resulting integration plasmid, pUCM1, was used to transform *A. naeslundii* WVU45 via electroporation (79). Transformants were selected on BHI plates containing kanamycin at a concentration of 50 μg/ml. A total of 66 kanamycin-resistant *A. naeslundii* strains were obtained, all of which had a urease-negative phenotype as determined by screening on urea agar/ supplemented with TH and kanamycin (described in Materials and Methods). This was the predicted phenotype for mutations occurring via either single or double recombination events.

Six of the transformants were randomly selected for further characterization by Southern blot analysis. Chromosomal DNAs from these strains were extracted, digested with *Bam*HI, and run on an agarose gel. The gel was then simultaneously transferred onto two nitrocellulose membranes, one of which was probed with a urease-specific probe, a 1.1-kbp *Bam*HI-*Sfi*I fragment of pUCM1, and the other of which was probed with the kanamycin resistance gene from pJRD215. Results for a single urease-negative strain, ANUC1, are presented here (Fig. 3B). The hybridization profile of this strain was consistent with insertion of the kanamycin resistance marker having occurred via a double-crossover recombination event. The same was true for one more of the six transformants that were subjected to Southern blot analysis, whereas the remaining four (66.7%) appeared to have occurred via single-crossover recombination (data not shown).

The kanamycin resistance gene was stably maintained in the chromosome of ANUC1 after passaging of the organism every day for 2 weeks in antibiotic-free medium (BHI broth). Also, the mutant strain exhibited a doubling time of approximately

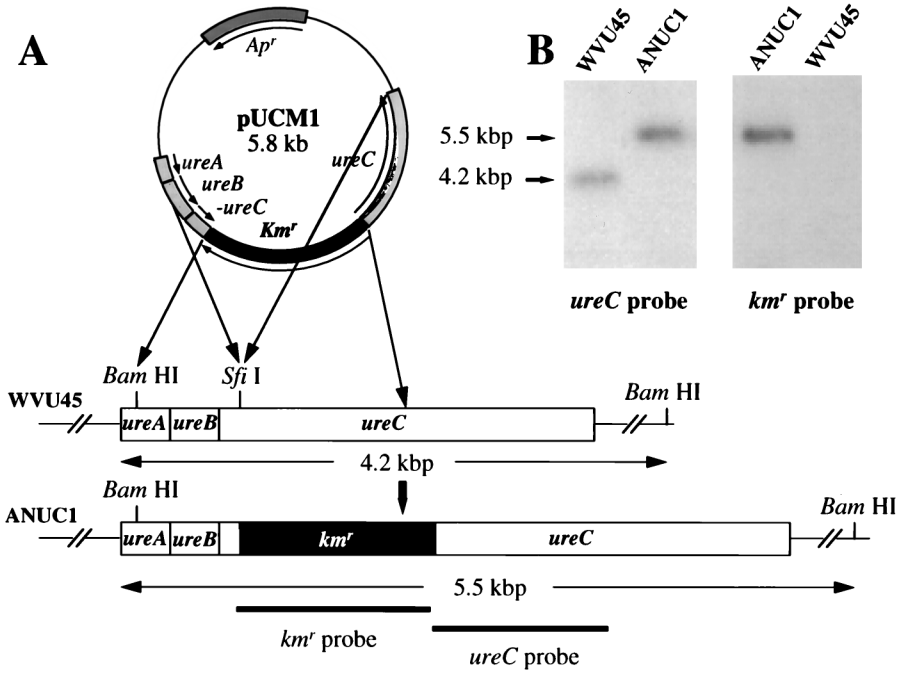

FIG. 3. (A) Schematic representation of the construction of the isogenic mutant, ANUC1. (B) Southern blot analysis of genomic DNA from the wild-type (WVU45) and urease-defective (ANUC1) *A. naeslundii* strains, probed with either a urease-specific probe or the Km^r gene. The locations of the probes on the chromosomes of the two strains are indicated in panel A.

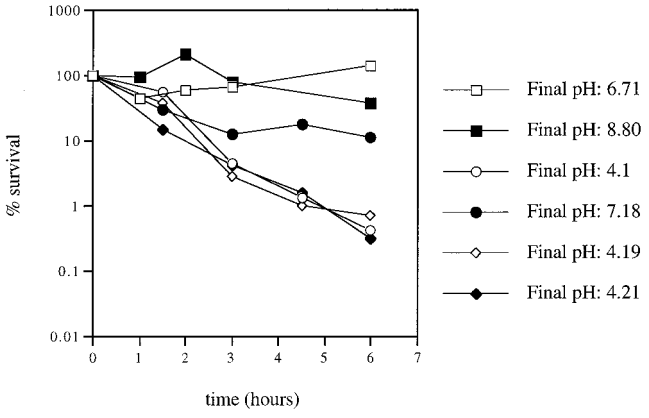

FIG. 4. Survival of *A. naeslundii* WVU45 or ANUC1 cells in citrate-phosphate buffer of pH 7.0 (squares [WVU45]) or 4.0 (circles [WVU45] and diamonds [ANUC1]) with (closed symbols) or without (open symbols) the addition of 25 mM urea. Cell viability at each time point is expressed as the percentage of the viable cells (CFU/milliliter of culture) at time zero. The pH value of each cell suspension at the end of the experiment (6 h) is indicated on the right. The data shown represent those from one of three individual experiments.

2.7 h, which is very comparable to that of the wild-type organism growing under the same conditions (data not shown).

Analysis of the functions of urease in *A. naeslundii*. Ureolysis has been shown to be protective against environmental acidification for organisms such as the gastrointestinal pathogen *Helicobacter pylori* (45) and the oral organism *Streptococcus salivarius* (59). We found that *A. naeslundii* was only moderately sensitive to environmental pH values of as low as 4.0, since it took longer than 6 h for a 4-log-unit reduction in the viability of cells to occur at this pH (Fig. 4). An equivalent decrease of cell viability took place within 2 to 3 h at pH 3.0 (data not shown). Addition of 25 mM urea conferred as much as a 100-fold increase in the survival of *A. naeslundii* WVU45, but not ANUC1, at pH 4.0 (Fig. 4), whereas at pH 3.0, ureolysis had no protective effect (data not shown). The pH values of the urea-containing wild-type cell suspensions at pH 7.0 or 4.0 increased by 1.5 to 3 pH units by the end of the experiment (Fig. 4), while the pH of the cell suspensions in buffer pH 3.0 remained unchanged (data not shown). These observations were consistent with our unpublished observations which indicated that the activity of the urease enzyme in intact cells of *A. naeslundii* decreases progressively as the pH drops from 6.0 (maximum activity) to 4.0, with no detectable urease activity at pH 3.0. It should be noted that the results of the acid-killing experiments were variable between experiments in terms of the lengths of time it took to achieve killing at a certain pH and the levels of protection conferred by urea. This seemed to be attributable to variations in the levels of urease activity between the different cultures and the tendency of the cells to clump at low pH values, which could affect the recovery of colonies. Despite this variability, it became clear after repeated performance of the experiment that ureolysis can be protective for *A. naeslundii* against killing at environmental pH values of as low as 4.0.

Another potentially critical role for urease in *A. naeslundii* could be to provide a source of assimilable nitrogen. In order to explore this hypothesis, we employed a semidefined medium (ADM) (7) to determine whether urea could serve as a nitrogen source for *A. naeslundii*. The basic solution of complete ADM contains 0.2% Casamino Acids and also contains phosphates, sodium, magnesium and calcium salts, glucose, and small amounts of cysteine, asparagine, tryptophane, glutathione, and glutamate. The basic solution was then diluted two-, four-, and eightfold, so that the amount of Casamino Acids was reduced to 0.1, 0.05, or 0.025%. The amount of glucose was maintained the same (0.5%) in all four dilutions of the medium. Cultures of *A. naeslundii* in complete ADM reached final optical densities at 600 nm (OD_{600}s) of 1.2. As the amount of Casamino Acids was reduced, the growth became more and more restricted, so that the final OD_{600} of the cultures in ADM containing 0.1% Casamino Acids was around 0.8, that in 0.05% Casamino Acids was 0.5, and the cultures in ADM containing 0.025% Casamino Acids never grew beyond an OD_{600} of 0.3 (Fig. 5). Supplementation with 25 mM NH_4Cl or 25 mM urea enhanced the growth of *A. naeslundii* in all dilutions of the medium. Increasing the concentration of ammonia to 50 mM, in order to account for the differences between the number of molecules of ammonia in urea and NH_4Cl, did not increase the yield, as defined by the final OD, of the corresponding cultures (data not shown). The cultures that were supplemented with urea consistently reached higher final OD_{600} values than those supplemented with NH_4Cl. However, as would be predicted, cultures grown in urea had substantially higher pH values than those grown in ammonia (Fig. 5).

To determine whether the increased final ODs of the cultures growing in urea compared to those growing in NH_4^+ were related to pH differences, growth curves in medium that had been buffered by the addition of 35 mM potassium phosphate were determined (Fig. 6). These and the following experiments were performed in ADM containing 0.05% Casamino Acids, because the differences in yields on the various nitrogen sources were most evident at this dilution and an acceptable growth could still be achieved even in the absence of nitrogen supplementation. Buffering alone did not enhance the growth of *A. naeslundii* in ADM containing 0.05% Casamino Acids, compared to that in the unbuffered medium, when no additional nitrogen source was provided. Cultures that were supplemented with 25 mM urea grew equally well regardless of whether the medium was buffered. When the medium was supplemented with either 25 mM NH_4Cl or 0.2% Casamino Acids (as in the complete medium), the buffered cultures achieved OD_{600} values higher than those of the unbuffered ones and comparable to those of the cultures that were supplemented with urea. Thus, it appeared that wild-type *A. naeslundii* could grow almost equally well in the presence of urea, ammonia, or Casamino Acids as a nitrogen source, when the pH was not a restricting factor.

The growth of the isogenic mutant in buffered ADM containing only 0.05% Casamino Acids or supplemented with 25 mM NH_4Cl or 0.2% Casamino acids was comparable to the growth of wild-type *A. naeslundii* under the same conditions (Fig. 7). In contrast to the wild-type organism, strain ANUC1 was not able to use urea for growth in this nitrogen-limited medium, which indicated that utilization of urea by *A. naeslundii* as a source of nitrogen occurs exclusively via a urease-dependent pathway.

DISCUSSION

Human dental plaque demonstrates high levels of ureolytic activity (60), which has been implicated in plaque pH homeostasis and plaque ecology and in the development of dental caries, calculus, and periodontal disease. Yet, the organisms that are primarily responsible for this activity have not been unequivocally identified, and molecular aspects of ureolysis in dental plaque remain largely unexplored. Of the dental plaque organisms that demonstrate ureolytic activity on isolation, *Actinomyces* strains are found in significant numbers in both

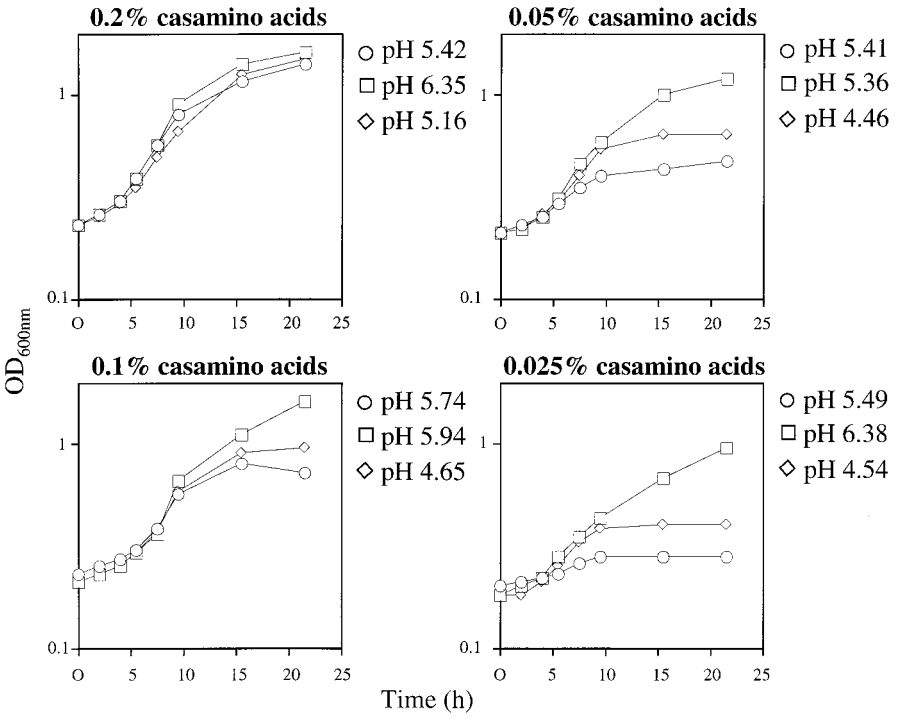

FIG. 5. Growth of wild-type *A. naeslundii* in ADM (see text) containing 0.2, 0.1, 0.05, or 0.025% Casamino Acids with either no additional nitrogen source (○) or supplemented with either 25 mM urea (□) or 25 mM ammonium chloride (◇). Numbers at the top right corner of each graph indicate final pH values of the cultures. The data are representative of those from six individual experiments.

supragingival and subgingival plaque and therefore have the potential to be important contributors to total plaque ureolysis (74).

Most bacterial urease operons are generally similar in their organization, being comprised of structural genes, genes involved in the incorporation of nickel into the apoenzyme, and in some cases genes involved in nickel uptake or regulation of urease expression. Differences between species usually occur in the arrangement of the genes and the spacing between them (16, 46). The gene order in the urease gene cluster of *A. naeslundii* was more similar to those of *S. salivarius* 57.I (11) and *Bacillus* species strain TB-90 (37), in that *ureD* was located in the 3′ end of the cluster, rather than the 5′ end as in some other organisms. Some urease clusters contain additional urease-related genes, such as the *Bacillus ureH* gene, which is thought to be involved in nickel transport (37), or the *H. pylori* and *S. salivarius ureI* genes, which have a function that is yet to be established (11, 18). No ORFs with homology to any of those genes were identified in the regions immediately 5′ to *ureA* or 3′ to *ureD* of *A. naeslundii*.

The 1-kbp region 5′ to the coding sequence of *ureA* contains three putative ORFs transcribed in the same direction as the urease genes. The most distant of these ORFs (ORF L31) is obviously not related to known urease genes, since it is highly homologous to genes encoding 50S ribosomal proteins (L31). It seems unlikely that the remaining two ORFs are translated in vivo, because of their small size and the lack of homology of their deduced amino acid sequences to known proteins. It appears, therefore, that no additional ORFs that could be cotranscribed with the urease genes exist within the 500-bp region between ORF L31 and the urease cluster. The transcriptional initiation site for the urease genes was mapped in this region, at 66 bases 5′ to the start codon for *ureA*. Given the observations that the urease genes are tightly spaced and that there is a lack of potential Rho-independent terminators in the intergenic regions, it is possible that these genes constitute an operon, similar to the case for numerous other urease gene clusters. We have made several attempts, using multiple RNA isolation procedures and growth conditions, to identify transcripts with *ure* gene probes. These efforts have been impeded by the comparatively low abundance and apparent short half-life of the corresponding transcripts and have been further complicated by substantial degradation of the RNA, which is

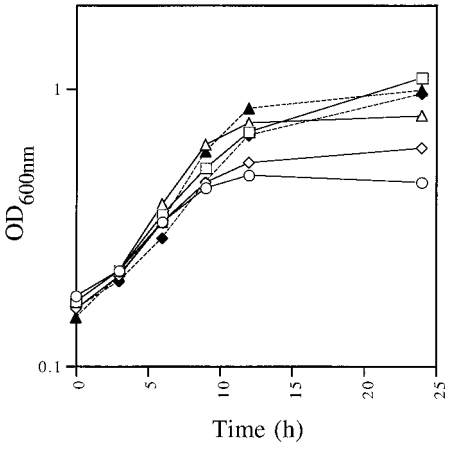

FIG. 6. Growth of wild-type *A. naeslundii* in ADM (see text) containing only 0.05% Casamino Acids as a nitrogen source (○) or supplemented with either 25 mM urea (□), 25 mM ammonium chloride (◇), 25 mM ammonium chloride plus 35 mM potassium phosphate buffer (pH 7.0) (◆), 0.2% Casamino Acids (△), or 0.2% Casamino Acids plus 35 mM potassium phosphate buffer (pH 7.0) (▲). The data shown represent those from one of six individual experiments.

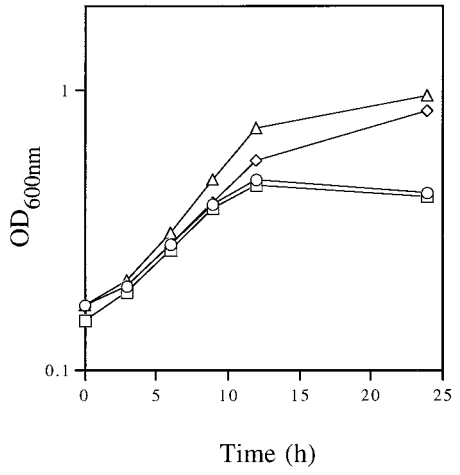

FIG. 7. Growth of the urease-defective strain ANUC1 in ADM containing 0.05% Casamino Acids and buffered with 35 mM potassium phosphate buffer (pH 7.0) without an additional nitrogen source (○) or supplemented with 25 mM urea (□), 25 ammonium chloride (◇), or 0.2% Casamino Acids (△). The data shown represent those from one of six individual experiments.

typical of a variety of oral bacteria. Thus, at this time we have no definitive answer as to whether all or some of the *A. naeslundii ure* genes are cotranscribed.

Currently, our knowledge of the characteristics and functions of promoters in *Actinomyces* is extremely limited, but it has been suggested that this species may possess a distinct class of promoter sequences (75). The promoter region of the *A. naeslundii* urease gene cluster has some similarities to known promoters from *Actinomyces* or the related genus *Streptomyces*, such as (i) an unusually high frequency of adenine and thymine phosphonucleotide residues, which is also observed in the *A. naeslundii* T14V *levJ* promoter (51) and in several *Streptomyces* promoters (68), and (ii) the presence of sequences with homology to *E. coli* σ^{70} promoters, which is also observed in the region 5′ to the *A. viscosus* T14V *nanH* gene (75) and in a number of *Streptomyces* promoters (68). Although many of the promoters that possess these characteristics appear to be functional in *E. coli*, it is still possible that they may not have activity in vivo in their original hosts (68, 75). The possibility that the sequence TATAA present in the *A. naeslundii* urease promoter region functions as the recognition site for the *A. naeslundii* RNA polymerase in vivo seems unlikely, since based on our primer extension data, this sequence is situated at position −4 with respect to the transcription initiation site. This would violate the strict spacing requirements for transcription initiation by the σ^{70}-RNA polymerase complex. Interestingly, the −10 sequence of the *A. naeslundii* urease promoter region is similar to the −12 consensus recognition site for RNA polymerase associated with σ^{54}, which is frequently involved in transcription of nitrogen-regulated genes. Functional studies to further characterize the promoter of the *A. naeslundii* urease gene cluster have been initiated.

Our data indicate that the physiological significance of urease in *A. naeslundii* is to provide a source of assimilable nitrogen and to confer some degree of protection against environmental acidification within a comparatively narrow range of clinically relevant pH values. Cultures that utilized urea as a nitrogen source always reached higher ODs than those growing on ammonia or a mixture of amino acids. The higher ODs of the cultures with urea were invariably associated with more neutral pH values than those found in the cultures that utilized other nitrogen sources. The explanation for these observations is likely to have two components. First, the utilization of urea may be bioenergetically favorable, since urea is an uncharged molecule which, unlike the NH_4^+ ion (35), probably does not require energy for transport into the cell. Second, once inside the cytoplasm, each molecule of urea can generate two molecules of ammonia, which in contrast to exogenously supplied ammonium ion, can become protonated and thus alkalinize the cytoplasm (45). Urease activity did not appear to protect *A. naeslundii* against killing at a pH of ≤3.0, yet it considerably increased its survival at pH 4.0. Although the exact levels of protection against environmental acidification provided by ureolysis were difficult to determine due to problems reported in Results, our data suggest that urease may confer to *A. naeslundii* protection from acid-induced damage during growth at clinically relevant pH values, i.e., in a range between 4.0 and 7.5.

The ability of *A. naeslundii* to utilize urea as a nitrogen source and as a means of protection against environmental acidification could constitute an important ecological determinant in dental plaque, especially when fermentable carbohydrates are present in excess. A carbohydrate-rich diet is known to promote the overgrowth of acidogenic mutans streptococci and lactobacilli in dental plaque, at the expense of less acidogenic organisms, such as *Streptococcus sanguis* and the *Actinomyces* spp. (4, 8, 10). The potential of plaque bacteria to withstand this major ecological pressure depends on their abilities to compete for nutrients and to survive in the acidic environment generated by the increased production of organic acids from glycolysis. The utilization of urea could confer to *A. naeslundii* two significant competitive advantages under these conditions. First, it would provide efficient access to a highly abundant nitrogen source, since urea is continuously supplied in saliva and gingival crevicular fluid at concentrations that range from 3 to 10 mM (28). This source is not available to the nonureolytic mutans streptococci, which have to rely heavily on the more limited amino acid and oligopeptide pools for protein synthesis. Second, as our data indicated, the production of ammonia from urea could neutralize organic acids produced during this period of increased glycolytic activity in plaque and render the environmental pH more favorable for itself and other less-acid-tolerant organisms (15). Since the balance between the acidogenic and the less aciduric organisms is one of the most important determinants of caries susceptibility (72), ureolysis by *A. naeslundii* could reduce the cariogenic potential of plaque by preventing the shift to a very acidogenic flora. The effects of the *A. naeslundii* urease in oral ecology could be even more significant during the early stages of colonization of the oral tissues, since *A. naeslundii* is one of the pioneer organisms in the oral cavity and is an early colonizer of the tooth surface.

In addition to it being one of the very first organisms to colonize the human oral cavity, one of the most important properties of *A. naeslundii* is probably its ability to successfully thrive both above and below the gingival margin. Supragingival and subgingival dental plaque constitute two distinct ecological niches, and the generation of ammonia from urea by *A. naeslundii* could have a totally different clinical impact in each location. This could possibly explain the controversial pathogenic profile of this organism. In supragingival plaque, ureolysis by *A. naeslundii* could modulate glycolytic acidification and inhibit dental caries, while the production of ammonia and elevation of pH by the same organism in subgingival plaque could promote calculus formation and periodontal inflammation. Our urease-defective strain, if able to be established in an appropriate animal model, could be very useful in helping us to understand the potential contribution of the *A. naeslundii*

urease in oral ecology, dental caries prevention, and possibly the formation of calculus and the pathogenesis of periodontal diseases.

ACKNOWLEDGMENTS

We thank Maria K. Yeung (University of Texas Health Science Center at San Antonio), George H. W. Bowden (University of Manitoba), and Margaret Chen (University of Rochester) for providing bacterial strains, plasmids, and/or technical advice. We also thank Margaret Chen for critical evaluation of the manuscript.

This work was supported by National Institute of Dental Research grants RO1 DE10362 and T32 DE07165.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Short protocols in molecular biology. Wiley Interscience, New York, N.Y.
2. **Birnboim, H. C., and L. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
3. **Biswas, S., D. F. Duperon, and F. S. Chebib.** 1977. Study of periodontal disease in children and young adolescents. I. Effect of age, sex, and gingival inflammation on crevice fluid volume, pocket depth, pH of supragingival plaque and crevice, collagenase activity and urea. J. Periodontal Res. **12:**250–264.
4. **Bowden, G. H., D. C. Ellwood, and I. R. Hamilton.** 1979. Microbial ecology of the oral cavity, p. 135–217. *In* M. Alexander (ed.), Advances in microbial ecology, vol. 3. Plenum Press, New York, N.Y.
5. **Bowden, G. H., J. M. Hardie, and G. L. Slack.** 1975. Microbial variations in approximal dental plaque. Caries Res. **9:**253–277.
6. **Bowden, G. H. W.** 1990. Microbiology of root surface caries in humans. J. Dent. Res. **69:**1205–1210.
7. **Bowden, G. H. W., J. M. Hardie, and E. D. Fillery.** 1976. Antigens from *Actinomyces* species and their value in identification. J. Dent. Res. **55**(Spec. Iss. A)**:**192–204.
8. **Bradshaw, D. J., A. S. McKee, and P. D. Marsh.** 1989. Effects of carbohydrate pulses and pH on population shifts within oral microbial communities. J. Dent. Res. **68:**1298–1302.
9. **Buck, G. E.** 1990. *Campylobacter pylori* and gastroduodenal disease. Clin. Microbiol. Rev. **3:**1–12.
10. **Burne, R. A.** 1998. Oral *streptococci*. . . products of their environment. J. Dent. Res. **77:**445–452.
11. **Chen, Y., K. A. Clancy, and R. A. Burne.** 1996. *Streptococcus salivarius* urease: genetic and biochemical characterization and expression in a dental plaque streptococcus. Infect. Immun. **64:**585–592.
12. **Chen, Y. M., and R. A. Burne.** 1995. Molecular analysis of the urease of *Streptococcus salivarius* 57.I. Dev. Biol. Stand. **85:**387–392.
13. **Chisolm, D.** 1989. A convenient moderate-scale procedure for obtaining DNA from bacteriophage lambda. BioTechniques **7:**21–23.
14. **Cisar, J. O., P. E. Colenbrander, and F. C. McIntire.** 1979. Specificity of coaggregation reactions between human oral streptococci and strains of *Actinomyces viscosus* or *Actinomyces naeslundii*. Infect. Immun. **24:**742–752.
15. **Clancy, K. A., and R. A. Burne.** 1997. Construction and characterization of a recombinant ureolytic *Streptococcus mutans* and its use to demonstrate the relationship of urease activity to pH modulating capacity. FEMS Microbiol. Lett. **151:**205–211.
16. **Collins, C. M., and S. E. F. Dorazio.** 1993. Bacterial ureases: structure, regulation of expression and role in pathogenesis. Mol. Microbiol. **9:**907–913.
17. **Cruz-Ramos, H., P. Glaser, J. L. V. Wray, and S. H. Fisher.** 1997. The *Bacillus subtilis ureABC* operon. J. Bacteriol. **179:**3371–3373.
18. **Cussac, V., R. L. Ferrero, and A. Labigne.** 1992. Expression of *Helicobacter pylori* urease genes in *Escherichia coli* grown under nitrogen-limiting conditions. J. Bacteriol. **174:**2466–2473.
19. **Davison, J., M. Heusterpreute, N. Hevalier, H.-T. Vinh, and F. Brunel.** 1987. Vectors with restriction site banks. V. pJRD215, a wide host range cosmid vector with multiple cloning sites. Gene **51:**275–280.
20. **Donkersloot, J. A., J. O. Cisar, M. E. Wax, R. J. Harr, and B. M. Chassy.** 1985. Expression of *Actinomyces viscosus* antigens in *Escherichia coli*: cloning of a structural gene (*fimA*) for type 2 fimbriae. J. Bacteriol. **162:**1075–1078.
21. **D'Orazio, S. E, and C. M. Collins.** 1993. The plasmid-encoded urease gene cluster of the family *Enterobacteriaceae* is positively regulated by UreR, a member of the AraC family of transcriptional activators. J. Bacteriol. **175:**3459–3467.
22. **Efthymiou, C., and C. A. Hansen.** 1962. An antigenic analysis of *Lactobacillus acidophilus*. J. Infect. Dis. **110:**258–267.
23. **Elder, B., and G. H. Nancollas.** 1993. Physical and chemical aspects of the mineralization and demineralization of tooth components, p. 187–196. *In* W. H. Bowen and L. A. Tabak (ed.), Cariology for the nineties. University of Rochester Press, Rochester, N.Y.
24. **Ellen, R. P.** 1976. Establishment and distribution of *Actinomyces viscosus* and *Actinomyces naeslundii* in the human oral cavity. Infect. Immun. **14:**1119–1124.
25. **Epstein, S. R., I. Mandel, and I. W. Scopp.** 1980. Salivary composition and calculus formation in patients undergoing hemodialysis. J. Periodontol. **51:**336–338.
26. **Gallagher, I. H. C., E. I. F. Pearce, and E. M. Hancock.** 1984. The ureolytic microflora of immature dental plaque before and after rinsing with a urea-based mineralizing solution. J. Dent. Res. **63:**1037–1039.
27. **Garant, P. R.** 1976. Light and electron microscopic observations of osteoclastic alveolar bone resorption in rats monoinfected with *Actinomyces naeslundii*. J. Periodontol. **47:**717–723.
28. **Golub, L. M., S. M. Borden, and I. Kleinberg.** 1971. Urea content of gingival crevicular fluid and its relationship to periodontal disease in humans. J. Periodonal Res. **6:**243–251.
29. **Helgeland, K.** 1985. pH and the effect of NH_4Cl on human gingival fibroblasts. Scand. J. Dent. Res. **93:**39–45.
30. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
31. **Jabri, E., M. B. Carr, R. P. Hausinger, and P. A. Karplus.** 1995. The crystal structure of urease from *Klebsiella aerogenes*. Science **268:**998–1004.
32. **Jahns, T.** 1996. Ammonium/urea-dependent generation of a proton electrochemical potential and synthesis of ATP in *Bacillus pasteurii*. J. Bacteriol. **178:**403–409.
33. **Johnson, J. L., L. V. H. Moore, B. W. Kaneko, and E. C. Moore.** 1990. *Actinomyces georgiae* sp. nov., *Actinomyces gerencseriae* sp. nov., designation of two genospecies of *Actinomyces naeslundii*, and inclusion of *A. naeslundii* serotypes II and III and *Actinomyces viscosus* serotype II in *A. naeslundii* genospecies 2. Int. J. Syst. Bacteriol. **40:**273–286.
34. **Kleinberg, I.** 1967. Effect of urea concentrations on human plaque pH in *situ*. Arch. Oral Biol. **12:**1475–1484.
35. **Kleiner, D.** 1985. Bacterial ammonia transport. FEMS Microbiol. Rev. **32:**87–100.
36. **Lee, M. H., S. B. Mulrooney, M. J. Renner, Y. Markowitz, and R. P. Hausinger.** 1992. *Klebsiella aerogenes* urease gene cluster: sequence of *ureD* and demonstration that four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are involved in nickel metallocenter biogenesis. Protein Sci. **2:**1042–1052.
37. **Maeda, M., M. Hidaka, A. Nakamura, H. Masaki, and T. Uozumi.** 1994. Cloning, sequencing, and expression of thermophilic *Bacillus* sp. strain TB-90 urease gene complex in *Escherichia coli*. J. Bacteriol. **176:**432–442.
38. **Mandel, I. D., and R. H. Thompson.** 1967. The chemistry of parotid and submaxillary saliva in heavy calculus formers and non-formers. J. Periodontol. **38:**310–315.
39. **Mann, H. L., S. B. Mulrooney, M. J. Renner, Y. Markowicz, and R. P. Hausinger.** 1992. *Klebsiella aerogenes* urease gene cluster: sequence of *ureD* and demonstration that four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are involved in nickel metallocenter biosynthesis. J. Bacteriol. **174:**4324–4330.
40. **Martin, P. R., and R. P. Hausinger.** 1992. Site-directed mutagenesis of the active site cysteine in *Klebsiella aerogenes* urease. J. Biol. Chem. **267:**22024–22027.
41. **McKnight, S. L., and R. Kingsbury.** 1982. Transcription control signals of a eukaryotic protein-coding gene. Science **217:**316–324.
42. **McLean, R. J. C., J. C. Nickel, K.-J. Cheng, and J. W. Costerton.** 1988. The ecology and pathogenicity of urease-producing bacteria in the urinary tract. Crit. Rev. Microbiol. **16:**37–79.
43. **Meyerowitz, C.** 1993. Caries in renal dialysis patients, p. 249–260. *In* W. H. Bowen and L. A. Tabak (ed.), Cariology for the nineties. University of Rochester Press, Rochester N.Y.
44. **Mobley, H. L. T., R. M. Garner, and P. Bauerfeld.** 1995. *Helicobacter pylori* nickel-transport gene *nixA*: synthesis of catalytically active urease in *Escherichia coli* independent of growth conditions. Mol. Microbiol. **16:**97–109.
45. **Mobley, H. L. T., and R. P. Hausinger.** 1989. Microbiol ureases: significance, regulation, and molecular characterization. Microbiol. Rev. **53:**85–108.
46. **Mobley, H. L. T., M. D. Island, and R. P. Hausinger.** 1995. Molecular biology of ureases. Microbiol. Rev. **59:**451–480.
47. **Moncrief, M. B. C., and R. P. Hausinger.** 1997. Characterization of UreG, identification of a UreD-UreF-UreG complex, and evidence suggesting that a nucleotide-binding site in UreG is required for in vivo metallocenter assembly of *Klebsiella aerogenes* urease. J. Bacteriol. **179:**4081–4086.
48. **Mulrooney, S. B., and R. P. Hausinger.** 1990. Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. J. Bacteriol. **172:**5837–5843.
49. **Musher, D. M., D. P. Griffith, D. Yawn, and R. D. Rossen.** 1975. Role of urease in pyelonephritis resulting from urinary tract infection with *Proteus*. J. Infect. Dis. **131:**177–181.
50. **Nicholson, E. B., E. A. Concaugh, P. A. Foxall, M. D. Island, and H. L. Mobley.** 1993. *Proteus mirabilis* urease: transcriptional regulation by UreR. J. Bacteriol. **175:**465–473.
51. **Norman, J. M., K. L. Bunny, and P. M. Giffard.** 1995. Characterization of *levJ*, a sucrase/fructanase-encoding gene from *Actinomyces naeslundii* T14V,

and comparison of its products with other sucrose-cleaving enzymes. Gene **152:**93–98.
52. **Park, I. S., M. B. Carr, and R. P. Hausinger.** 1994. In vitro activation of urease apoprotein and role of UreD as a chaperone required for nickel metallocenter assembly. Proc. Natl. Acad. Sci. USA **91:**3233–3237.
53. **Park, I. S., and R. P. Hausinger.** 1993. Site-directed mutagenesis of *Klebsiella aerogenes* urease—identification of histidine residues that appear to function in nickel ligation, substrate binding, and catalysis. Protein Sci. **2:**1034–1041.
54. **Peterson, S., J. Woodhead, and J. Crall.** 1985. Caries resistance in children with chronic renal failure: plaque pH, salivary pH, and salivary composition. Pediatr. Res. **19:**796–799.
55. **Salako, N. O., and I. Kleinberg.** 1989. Incidence of selected ureolytic bacteria in human dental plaque from sites with different salivary access. Arch. Oral Biol. **34:**787–791.
56. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
57. **Shakespeare, A. P., D. B. Drucker, and R. M. Green.** 1985. The comparative cariogenicity and plaque-forming ability in vivo of four species of the bacterium *Actinomyces* in gnotobiotic rats. Arch. Oral Biol. **30:**855–858.
58. **Sissons, C. H., and T. W. Cutress.** 1988. pH changes during the simultaneous metabolism of urea and carbohydrate by human salivary bacteria *in vitro*. Arch. Oral Biol. **33:**579–587.
59. **Sissons, C. H., and E. M. Hancock.** 1993. Urease activity in *Streptococcus salivarius* at low pH. Arch. Oral Biol. **38:**507–516.
60. **Sissons, C. H., E. M. Hancock, and T. W. Cutress.** 1988. The source and variation in ureolysis in artificial plaques cultured from human salivary bacteria. Arch. Oral Biol. **33:**721–726.
61. **Sissons, C. H., E. M. Hancock, H. E. R. Perinpanayagam, and T. W. Cutress.** 1988. The bacteria responsible for ureolysis in artificial dental plaque. Arch. Oral Biol. **33:**727–734.
62. **Smith, D. G., W. C. Russel, W. J. Ingledew, and D. Thirkell.** 1993. Hydrolysis of urea by *Ureaplasma urealyticum* generates a transmembrane potential with resultant ATP synthesis. J. Bacteriol. **175:**3253–3258.
63. **Socransky, S. S., C. Hubersak, and D. Propas.** 1970. Induction of periodontal distraction in gnotobiotic rats by a human strain of *Actinomyces naeslundii*. Arch. Oral Biol. **15:**993–995.
64. **Socransky, S. S., and S. Manganiello.** 1971. The oral microbiota of man from birth to senility. J. Periodontol. **42:**485–496.
65. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
66. **Sriwanthana, B., M. D. Island, D. Maneval, and H. L. Mobley.** 1994. Single-step purification of *Proteus mirabilis* urease accessory protein UreE, a protein with a naturally occurring histidine tail, by nickel chelate affinity chromatography. J. Bacteriol. **176:**6836–6841.
67. **Sriwanthana, B., M. D. Island, and H. L. T. Mobley.** 1993. Sequence of the *Proteus mirabilis* urease accessory gene *ureG*. Gene **129:**103–106.
68. **Strohl, W. R.** 1992. Compilation and analysis of DNA sequences associated with apparent streptomyces promoters. Nucleic Acids Res. **20:**961–974.
69. **Syed, S. A., W. J. Loesche, H. L. Pape, and E. Grenier.** 1975. Predominant cultivable flora isolated from human root surface caries plaque. Infect. Immun. **11:**727–731.
70. **Syrrakou, E. P., and C. Meyerowitz.** 1995. Acid content and pH of plaque from renal disease patients. J. Dent. Res. **74:**48.
71. **Todd, M. J., and R. P. Hausinger.** 1991. Identification of the essential cysteine residue in *Klebsiella aerogenes* urease. J. Biol. Chem. **266:**24327–24331.
72. **Van Houte, J.** 1994. Role of micro-organisms in caries etiology. J. Dent. Res. **73:**672–681.
73. **Van Houte, J., J. Lopman, and R. Kent.** 1994. The predominant cultivable flora of sound and carious human root surfaces. J. Dent. Res. **73:**1727–1734.
74. **Wijeyeweera, R. L., and I. Kleinberg.** 1989. Arginolytic and ureolytic activities of pure cultures of human oral bacteria and their effects on the pH response of salivary sediment and dental plaque *in vitro*. Arch. Oral Biol. **34:**43–55.
75. **Yeung, M. K.** 1993. Complete nucleotide sequence of the *Actinomyces viscosus* T14V sialidase gene: presence of a conserved repeating sequence among strains of *Actinomyces* spp. Infect. Immun. **61:**109–116.
76. **Yeung, M. K.** 1992. Conservation of an *Actinomyces viscosus* T14V type 1 fimbrial subunit homologue among divergent groups of *Actinomyces* spp. Infect. Immun. **60:**1047–1054.
77. **Yeung, M. K.** 1995. Construction and use of integration plasmids to generate site-specific mutations in the *Actinomyces viscosus* T14V chromosome. Infect. Immun. **63:**2924–2930.
78. **Yeung, M. K., and J. O. Cisar.** 1988. Cloning and nucleotide sequence of a gene for *Actinomyces naeslundii* WVU45 type 2 fimbriae. J. Bacteriol. **170:**3803–3809.
79. **Yeung, M. K., and C. S. Kozelsky.** 1994. Transformation of *Actinomyces* spp. by a gram-negative broad-host-range plasmid. J. Bacteriol. **176:**4173–4176.

Editor: D. L. Burns