Identification and Characterization of the Nickel Uptake System for Urease Biogenesis in *Streptococcus salivarius* 57.I

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Ureases are multisubunit enzymes requiring Ni$^{2+}$ for activity. The low pH-inducible urease gene cluster in *Streptococcus salivarius* 57.I is organized as an operon, beginning with *ureI*, followed by *ureABC* (structural genes), and *ureEFGD* (accessory genes). Urease biogenesis also requires a high-affinity Ni$^{2+}$ uptake system. By searching the partial genome sequence of a closely related organism, *Streptococcus thermophilus* LMG18311, three open reading frame (ORFs) homologous to those encoding proteins involved in cobalamin biosynthesis and cobalt transport (chiM/QO) were identified immediately 3' to the *ure* operon. To determine whether these genes were involved in urease biogenesis by catalyzing Ni$^{2+}$ uptake in *S. salivarius*, regions 3' to *ureD* were amplified by PCRs from *S. salivarius* by using primers identical to the *S. thermophilus* sequences. Sequence analysis of the products revealed three ORFs. Reverse transcriptase PCR was used to demonstrate that the ORFs are transcribed as part of the *ure* operon. Insertional inactivation of *ORF1* with a polar kanamycin marker completely abolished urease activity and the ability to accumulate $\Delta^{3}$Ni$^{2+}$ during growth. Supplementation of the growth medium with NiCl$_2$ at concentrations as low as 2.5 $\mu$M partially restored urease activity in the mutant. Both wild-type and mutant strains showed enhanced urease activity when exogenous Ni$^{2+}$ was provided at neutral pH. Enhancement of urease activity by adding nickel was regulated at the posttranslational level. Thus, *ORF1*, *ORF2*, and *ORF3* are part of the *ure* operon, and these genes, designated *ureM*, *ureO*, and *ureQ*, respectively, likely encode a Ni$^{2+}$-specific ATP-binding cassette transporter.

Ureases are Ni$^{2+}$-requiring metallo-enzymes that have been isolated and characterized from a variety of prokaryotes and eukaryotes (21). The production of ammonia and CO$_2$ from urea hydrolysis by urease has been shown to have a major impact on microbial pathogenesis (4). Genes required for the biogenesis of bacterial ureases are generally arranged as operons, with the structural genes: *ureC*, *ureB*, and *ureA*, encoding the $\alpha$, $\beta$, and $\gamma$ subunits, respectively, followed by the accessory genes: *ureE*, *ureF*, *ureG*, and *ureD*, encoding proteins essential for the incorporation of Ni$^{2+}$ into the metallocenter. Other genes, such as *ureI*, encoding urea transporters, are found in the urease operons of *Helicobacter pylori* and *Streptococcus salivarius* (9, 29). Although Ni$^{2+}$ is an essential cofactor for the catalytic activity of urease, most known *ure* operons do not contain genes encoding proteins for Ni$^{2+}$ transportation. One possible exception is *ureH* of *Bacillus* sp. strain TB-90 (20), which shares homology with the high-affinity nickel-specific permease encoded by *hoxN* of *Ralsotinia eutropha* (12).

Nickel is usually present in trace amounts in the natural environment and is crucial for a number of biological processes, such as hydrolysis of urea, consumption of molecular hydrogen, and methanogenesis. Free nickel is toxic (28); therefore, nickel-specific transporters usually display high affinity but low capacity, presumably to guard against potential toxic effects caused by high levels of intracellular nickel. Thus far, two distinct high-affinity nickel transport systems have been described in prokaryotes: the single-component Ni$^{2+}$ transporters, which belong to the nickel/cobalt transporter (NiCoT) family, and the Nik systems, which belong to the ATP-dependent binding cassette (ABC) transporter family (14). The most studied, single-component Ni$^{2+}$-specific permease is *HoxN* from *R. eutropha* (13, 15, 30). Similar systems have been identified in other bacteria, including *HupN* from *Bradyrhizobium japonicum* (16), *Nixa* from *H. pylori* (17), and possibly *UreH* from *Bacillus* sp. strain TB-90 (20).

The Nik system was originally identified in *Escherichia coli* and is composed of one periplasmic Ni$^{2+}$ binding protein (NikA), two hydrophobic transmembrane proteins, NikB and NikC, which are assumed to form the channel for Ni$^{2+}$ uptake, and two membrane-associated components, NikD and NikE, which contain the conserved signature sequences of ATPases and are believed to be involved in the energy-coupling process for transport (22). The expression of the *E. coli nik* operon is negatively regulated at the transcriptional level by the NikR repressor protein when intracellular nickel levels are high (11). Similar Ni$^{2+}$ uptake systems have been identified in *Brucella suis* (18), *Vibrio paradoxum* (23), *Actinobacillus pleuro-pneumoniae* (2), and *Yersinia pseudotuberculosis* (25), and in all cases this Ni$^{2+}$-specific transporter is associated with ureolytic activity of the microorganisms.

*S. salivarius* is one of the most abundant and highly ureolytic microorganisms in the oral cavity and can use urea as a primary source of nitrogen (5). The expression of urease in *S. salivarius* is subject to environmental signals, with higher levels of expression in cells grown under acidic conditions, and the induction at acidic pH values can be enhanced by growth in excess amounts of carbohydrate (6). It is believed that regulation by low pH and carbohydrate availability allows for maximal production of the enzyme when it is most needed for survival of the organisms against lethal acidification (5). The urease gene...
cluster \textit{(ure)} of \textit{S. salivarius} is arranged as an operon, beginning with \textit{ureI}, followed by \textit{ureABCEFGD} \textit{(9)}. Previous studies indicated that \textit{ureABCEFGD} are required for assembly of a functional urease. However, a recombinant \textit{Streptococcus mutans} strain (ACUS6) harboring only the 3' half of \textit{ureI} by PCRs with primers derived from the \textit{ureM} operon and \textit{ureQ} insertion; II, products generated with a primer pair located 500 bp 3' to the ATG start codon of \textit{ureM}. In lanes 1 and 2, RT was included in the reactions; lanes 3 and 4 were controls reactions that were carried out identically to the experimental samples, but without RT. In lane 7, PCR was used to amplify the target region from \textit{S. salivarius} 57.1 chromosomal DNA. Lanes 1 and 4, products generated from cells grown in BHI-KPB; lanes 2 and 5, products from cells grown in BHI; lanes 3 and 6, products from cells grown in BHI-HCl. The 100-bp DNA ladder (MBI Fermentas) was used as the molecular weight marker. (C) PCR products generated from RT-PCR. A total of 10% of the total cDNA generated by RT-PCR from each RNA sample was amplified with specific primers (see panel A), and 10% of the total PCR products were run on a 0.8% Tris-acetate-EDTA gel. Subpanels: I, PCR products generated with a primer pair specific for the \textit{ureM} intergenic region; II, products generated with a primer pair specific for the \textit{ureOQ} intergenic region. In lanes 1, 2, and 3 RT was included in the reactions; lanes 4, 5, and 6 show the results control reactions that were carried out identically to the experimental samples, but without RT. PCR products generated from RT-PCR. A total of 10% of the total cDNA generated by RT-PCR from each RNA sample was amplified with specific primers (see panel A), and 10% of the total PCR products were run on a 0.8% Tris-acetate-EDTA gel. Subpanels: I, PCR products generated with a primer pair specific for the \textit{ureM} operon; II, products generated with a primer pair specific for the \textit{ureAB}; III, products generated with a primer pair specific for the \textit{ureI} gene (5): lanes 2 and 4, products from wild-type \textit{S. salivarius}. In lanes land 2, RT was included in the reactions; in lanes 3 and 4 were control reactions without RT. In lane 5, the PCR product was amplified from \textit{S. salivarius} 57.1 chromosomal DNA. Subpanels: I, PCR products generated with a pair primer specific for \textit{ureAB} region; II, products generated with a primer pair specific for the \textit{ureC}; III, products generated with a primer pair specific for the 3' half of \textit{ureM} (ORF1). Ladder mix DNA ladder (MBI Fermentas) was used as the molecular weight marker. (D) PCR products generated from RT-PCR. A total of 10% of total cDNA generated by RT-PCR from each RNA sample was amplified with specific primers (see panel A), and 20% of the total PCR products were run on a 0.8% Tris-acetate-EDTA gel. Subpanels: I, PCR products generated with a primer pair located immediately 3' to the \textit{Uk} insertion; II, products generated with a primer pair located 500 bp 3' to the \textit{ATG} start codon of \textit{ureM}. In lanes 1 and 2, RT was included in the reactions; lanes 3 and 4 were controls reactions without RT. In lane 5, the sample was PCR amplified from \textit{S. salivarius} 57.1 chromosomal DNA. The same DNA ladder was used as in panel B.

\textbf{Materials and Methods}

**Bacterial strains, growth conditions, and reagents.** \textit{S. salivarius} 57.1 and its \textit{UreMOQ}-deficient derivative were routinely grown in brain heart infusion (BHI; Difco Laboratories) at 37°C in 5% CO\textsubscript{2} and 95% air. Recombinant \textit{E. coli} strains were routinely maintained in LB broth. Kanamycin was included in the growth medium, when indicated, at 750 or 50 µg ml\textsuperscript{-1} for recombinant \textit{S. salivarius} or \textit{E. coli} strains, respectively. All chemical reagents and antibiotics were purchased from Sigma. 67NiCl\textsubscript{2} (9.87 mCi of Ni mg\textsuperscript{-1}) was purchased from Amersham Biosciences (Piscataway, N.J.). To obtain cultures grown under neutral or acidic conditions, cells were grown in BHI containing 50 mM potassium phosphate buffer (pH 7.4; BHI-KPB), BHI alone, or BHI that had been adjusted to pH 5.8 by the addition of 2 N HCl (BHI-HCl).

**Nucleic acid manipulations.** Genomic DNA from \textit{S. salivarius} 57.1 was isolated as previously described \textit{(8)}. Plasmid DNA from recombinant \textit{E. coli} strains was purified by using the QiAprep spin plasmid kit (Qiagen, Inc.). Cloning, Southern blot analysis, and hybridizations were carried out by using an established protocol \textit{(1)}. Restriction endonucleases, DNA polymerases, and RNA reverse transcriptase (RT) were obtained from Invitrogen or New England Biolabs (NEB). Total cellular RNA from \textit{S. salivarius} strains was isolated as described elsewhere \textit{(9)}. Levels of \textit{ure}-specific mRNA were quantitated by densitometry using slot blot analysis, with hybridization and washes carried out at high stringency \textit{(6)}.

**Isolation of \textit{ureM}, \textit{ureO}, and \textit{ureQ}.** The region immediately 3' to the \textit{ure} cluster was amplified from \textit{S. salivarius} 57.1 by PCRs with primers derived from the \textit{Streptococcus thermophilus} LMG18311 genome sequences (http://www.biol.ucd.id.ac.be/file/genenome/). PCR were initiated with five cycles at a less stringent annealing temperature (50°C), followed by 20 cycles at a more stringent annealing temperature (55°C). All PCR products were cloned onto plasmid pCRII (Invitrogen) and the sequences were determined.

**Construction of a \textit{UreMOQ}-deficient \textit{S. salivarius}.** A HindIII-Nei fragment, 2.9 kb in size, containing the 3' portion of \textit{ureG}, \textit{ureD} through \textit{ureM}, and the 5'
portion of ureQ (Fig. 1A) was initially subcloned from pMC11 (8) into pGEM3Zf(+) to generate plasmid pMC281. A DNA fragment containing a kanamycin resistance marker flanked by transcription/translation terminators (Pκron) (24) was subsequently cloned into the unique XhoI site, located at the beginning of ureM, on plasmid pMC281. The resulting chimeric plasmid, pMC282, was transferred into strain 57.1 by electroporation (9) to introduce a polar mutation in ureM by allelic exchange. The configuration of the double-crossover integration of the kanamycin resistance marker was confirmed by PCR and Southern blot analysis.

Nickel accumulation. A nickel accumulation assay was adopted from Wolfram et al. (30) with minor modifications. Briefly, overnight cultures of S. salivarius strains in BHI were diluted 1:50 into fresh BHI-KPB or BHI containing 500 nM nickel chloride. Unlabeled metal chlorides as competitors were added to a final concentration of 5 μM. All cultures were grown at 37°C for 5 h at which point the optical density at 600 nm (OD 600) of the cultures was ca. 0.9. Cells were harvested, washed twice with an equal volume of ice-cold buffer A (50 mM Tris-HCl, 10 mM MgCl2, pH 7.5), and then concentrated 40-fold in the same buffer. The radioactivity of an aliquot of the cell suspension (100 μl) was determined by liquid scintillation counting. To monitor the amount of 63Ni2+ accumulated intracellularly over time, cells were grown in BHI at 37°C for 3 h and 45 min prior to addition of 63NiCl2. A 1-ml aliquot of the cell suspension was harvested every 15 min after addition and processed as described above. The CFU count in the final concentration of nickel was determined by RT-PCR (Fig. 1C). When a polar mutation was introduced into ureC, no detectable ORF1-specific transcript was observed by RT-PCR, indicating ORF1, ORF2, and ORF3 were transcribed exclusively from the promoter 5′ to ureC. A polar insertion in ureM also resulted in no detectable ORF1 transcript by RT-PCR (data not shown). A stable stem-loop structure, with ΔG° = −10.3 kcal, followed by a stretch of six T residues, which could potentially function as a rho-independent terminator, was identified seven bases 3′ to the stop codon of ORF3, and no transcript could be detected by RT-PCR between ORF3 and the partial downstream ORF (data not shown), a finding indicating that this partial ORF is not part of the ure operon. The sequence of this partial ORF encoded a truncated peptide that shared significant homology (60 to 70% identity) with the N terminus of the substrate-binding proteins of amino acid ABC-type transporters (AtmA) from other streptococcal species. The lack of involvement of this ORF in urea metabolism was further confirmed by demonstrating that there was no change in urease activity in a recombinant strain in which this ORF was insertionally inactivated (data not shown). ORF1, ORF2, and ORF3 were designated ureM, ureQ, and ureO, respectively, and it was concluded that the ure operon of S. salivarius consists of 11 genes (ureA, B, C, D, E, F, G, M, Q, O, and I), the ureBOperon, and the expression of these operons is generally regulated independently from ure operons (2, 18).

Translation of S. salivarius ureM predicted a protein of 325 amino acids with a pI of 8.99 and a calculated mass of 35.2 kDa. UreM shared significant degrees of similarity with CbiM, which has been suggested to encode an integral membrane protein involved in cobalt transport for cobalamin biosynthesis, from Thermoanaerobacter tengcongensis (47% identity and 64% similarity) and Clostridium acetobutylicum (39% identity and 58% similarity). The conserved domain of the permease component of ABC-type C02+ transport systems could also be identified within UreM, and three membrane helices were predicted by using CBS prediction services (Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark [http://www.cbs.dtu.dk/services/]). S. salivarius ureQ (777 nucleotides) encoded a 29.1-kDa protein with a pI of 9.63. The highest degree of similarity was observed between ureQ and genes predicted to encode cobalt permeases from Lactobacillus plantarum (28% identity and 43% similarity) and from C. acetobutylicum (24% identity and 46% similarity). Eight membrane helices were predicted in deduced UreQ sequence by using CBS prediction services. S. salivarius ureO

**RESULTS AND DISCUSSION**

Isolation and sequence analysis of ureM, ureQ, and ureO. Urease biosynthesis by bacteria requires a high-affinity Ni2+ uptake system. By searching the partial genome sequence of a closely related, ureolytic organism, S. thermophilus LMG18311, three open reading frames (ORFs) related to those encoding proteins similar to the ABC-type cobalt transport system found in cobalamin biosynthesis operons (cbiMQO) were identified immediately 3′ to the ure operon. To determine whether these genes were present in S. salivarius, a chromosomal walking approach was used to obtain sequences 3′ to ureD by PCRs from S. salivarius with primers identical to the cbiM, cbiQ, and cbiO genes of S. thermophilus. Approximately 3 kbp of sequence immediately 3′ to ureD was obtained from the three overlapping PCR products. Three complete ORFs and an additional partial ORF, all in the same orientation as the ure operon, could be identified within this 3-kbp region. ORF1, with its ribosome binding site embedded in ureD, is located three bases 3′ to the stop codon of ureD. ORF2 overlaps with ORF1 by two bases, and ORF3 is located one base 3′ to ORF2. The partial ORF is located 165 bp 3′ to ORF3. To determine whether these ORFs were cotranscribed with the ure operon, RT-PCR was used to detect the existence of contiguous transcripts between ureD, ORF1, ORF2, and ORF3. The results indicated the presence of contiguous transcript(s) between ureD, ORF1, ORF2, and ORF3, suggesting that all three ORFs could be cotranscribed with the urease genes and were part of the ure operon (Fig. 1B). To determine whether ORF1, ORF2, and ORF3 could be transcribed independently, presumably via a promoter embedded somewhere within the ure-D genes, total cellular RNA was isolated from a recombinant S. salivarius strain in which ureC had been insertionally inactivated by allelic exchange (5), and the presence of ORF1-specific mRNA was determined by RT-PCR (Fig. 1C). When a polar mutation was introduced into ureC, no detectable ORF1-specific transcript was observed by RT-PCR, indicating ORF1, ORF2, and ORF3 were transcribed exclusively from the promoter 5′ to ureC. A polar insertion in ureM also resulted in no detectable ORF1 transcript by RT-PCR (data not shown). A stable stem-loop structure, with ΔG° = −10.3 kcal, followed by a stretch of six T residues, which could potentially function as a rho-independent terminator, was identified seven bases 3′ to the stop codon of ORF3, and no transcript could be detected by RT-PCR between ORF3 and the partial downstream ORF (data not shown), a finding indicating that this partial ORF is not part of the ure operon. The sequence of this partial ORF encoded a truncated peptide that shared significant homology (60 to 70% identity) with the N terminus of the substrate-binding proteins of amino acid ABC-type transporters (AtmA) from other streptococcal species. The lack of involvement of this ORF in urea metabolism was further confirmed by demonstrating that there was no change in urease activity in a recombinant strain in which this ORF was insertionally inactivated (data not shown). ORF1, ORF2, and ORF3 were designated ureM, ureQ, and ureO, respectively, and it was concluded that the ure operon of S. salivarius consists of 11 genes (ureA, B, C, D, E, F, G, M, Q, O, and I), the ureBOperon, and the expression of these operons is generally regulated independently from ure operons (2, 18).

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(714 nucleotides) encoded a 27.6-kDa protein with a pI of 5.44. UreO shared 44, 40, and 37% identity to the ATPase components of ABC-type cobalt transport systems (CbiO) of L. plantarum, C. acetobutylicum, and T. tengcongensis, respectively. Significant levels of similarity to the ATP-binding proteins of cobalt ABC transporters from Methanosarcina species were also observed (35% identity). The linker peptide (LSGGIEKKK), and the Walker A (GENGTGKS) and Walker B (VYILD) motifs commonly found in many ATP- and GTP-binding and hydrolyzing proteins were also observed in the correct respective positions in the protein.

**Functional analysis of UreMQO in Ni\(^{2+}\) accumulation.** To examine the impact of UreMQO in urease biosynthesis, a polar mutation (\(\delta\)kan) was introduced into ureM, four bases 3' to the ATG start codon. To ensure that the transcription of the urease operon terminated at the position of \(\delta\)kan, total RNA was isolated from S. salivarius wild-type and UreMQO-deficient strains, and RT-PCR was used to detect ureM-specific mRNA (Fig. 1D). No RT-PCR product could be detected 3' to \(\delta\)kan.

Earlier studies demonstrated that urease expression in S. salivarius is predominantly regulated by growth pH (6, 9). At neutral pH, expression is almost completely repressed. Induction occurs and increases as the growth pH becomes more acidic. To determine whether the capacity to accumulate Ni\(^{2+}\) in both wild-type and UreMQO-deficient strains was also regulated by pH, cells were cultured in BHI-KPB or in BHI as described in Materials and Methods to late-exponential phase, at which point the cultures were at approximately pH 7.0 and 5.5, respectively. When cells were incubated with 500 nM \(^{63}\)Ni\(^{2+}\), it was found that excess amounts of unlabeled Co\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\), and Ni\(^{2+}\) at a 10-fold excess (5 \(\mu\)M) in growth medium containing 500 nM \(^{63}\)Ni\(^{2+}\). It was found that excess amounts of unlabeled Co\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), or Mg\(^{2+}\) chloride did not result in a significant decrease in the amount of \(^{63}\)Ni\(^{2+}\) accumulated (Fig. 4). However, the inclusion of 5 \(\mu\)M unlabeled NiCl\(_2\) in the growth medium inhibited the accumulation confirmed by incubating the cells with different amounts of \(^{63}\)Ni\(^{2+}\), and it was found that the amount of intracellular \(^{63}\)Ni\(^{2+}\) was a direct result of the concentrations of exogenous of \(^{63}\)Ni\(^{2+}\) (Fig. 3).

The substrate specificity of UreMQO in wild-type S. salivarius was examined by the addition of unlabeled Co\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\), and Ni\(^{2+}\) at a 10-fold excess (5 \(\mu\)M) in growth medium containing 500 nM \(^{63}\)Ni\(^{2+}\). It was found that excess amounts of unlabeled Co\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), or Mg\(^{2+}\) chloride did not result in a significant decrease in the amount of \(^{63}\)Ni\(^{2+}\) accumulated (Fig. 4). However, the inclusion of 5 \(\mu\)M unlabeled NiCl\(_2\) in the growth medium inhibited the accumulation confirmed by incubating the cells with different amounts of \(^{63}\)Ni\(^{2+}\), and it was found that the amount of intracellular \(^{63}\)Ni\(^{2+}\) was a direct result of the concentrations of exogenous of \(^{63}\)Ni\(^{2+}\) (Fig. 3).

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of $^{63}\text{Ni}^{2+}$ by 85%, indicating that UreMQO have specificity for $\text{Ni}^{2+}$ ion.

Initial attempts to determine the kinetic parameter of UreMQO in nickel transport by using standard transport assays were unsuccessful, presumably due to the low transport capacity of the system and a high level of nonspecific binding of the $\text{Ni}^{2+}$ to the cell surface. To circumvent this limitation, we used a modified accumulation assay in a time course study, in which a larger size of cell suspension was collected by centrifugation at each time point, followed by two washes with buffer containing 10 mM Mg$^{2+}$ to remove nonspecifically bound $^{63}\text{Ni}^{2+}$. It was found that the expression of the $S$. salivarius urease operon, which is tightly regulated by growth pH (7), was repressed until the pH of the culture reached ~6.5, at which time the urease specific activity increased linearly and peaked during the late exponential phase of growth (Fig. 5A). To ensure that nickel accumulation was monitored during biogenesis of an active urease, $^{63}\text{Ni}^{2+}$ was added to the cultures at 3 h and 45 min postinoculation, and the amount of $^{63}\text{Ni}^{2+}$ was monitored every 15 min afterward. A time-dependent accumulation was observed, and the accumulation reached maximal levels 60 min after the addition of $^{63}\text{Ni}^{2+}$ (Fig. 5B). No significant uptake of nickel occurred prior to the induction of the urease operon (data not shown).

Urease activity was enhanced by exogenous $\text{NiCl}_2$. It is known that the average concentration of nickel in the natural environment is in the nanomolar range and that the most commonly occurring oxidation state of nickel is Ni(II). When Ni$^{2+}$ is present at higher concentrations, it can be transported by Mg$^{2+}$ transport systems (19, 26). To determine the influence of UreMQO in overall urease biosynthesis and whether high concentrations of exogenous Ni$^{2+}$ could compensate for the deficiency of the Ni$^{2+}$-specific uptake system, possibly through a Mg$^{2+}$ uptake system, urease activities were examined in wild-type and UreMQO-deficient strains at different growth pH values, with or without additional NiCl$_2$ (Fig. 6). No detectable urease activity was observed in the UreMQO-deficient strain in the absence of supplemented NiCl$_2$, regardless of the growth pH. When cells were grown at neutral pH, supplementation with as little as 2.5 $\mu$M NiCl$_2$ was able to partially restore the ureolytic phenotype in the UreMQO-deficient strain, and the levels of urease activity increased in a NiCl$_2$ concentration-dependent manner. NiCl$_2$-dependent increases in urease activity were also observed in cells grown in BHI and BHI-HCl, and the highest levels of urease activity at each NiCl$_2$ concentration supplied to the system were consistently observed in cells grown in BHI-HCl. In the absence of exogenous NiCl$_2$, urease activity in the wild-type strain was solely regulated by growth pH, with the highest urease activity observed in cells grown in BHI-HCl.

Interestingly, when cells were grown at neutral pH (BHI-KPB), urease activity in the wild-type cells increased in response to increasing concentrations of NiCl$_2$, and the enhancement of NiCl$_2$ reached saturation at 25 $\mu$M. The enhancement of urease activity by NiCl$_2$ was less prominent when cells were grown at acidic pH. When cells were grown in BHI without any buffer, the highest level of activation by NiCl$_2$ occurred at 2.5 $\mu$M, but there was no significant increase in urease activity with higher concentrations of NiCl$_2$ when cells were grown in BHI-HCl. Therefore, under conditions in which the ureMQO may not be fully induced, the addition of nickel can increase the amount of activated urease, suggesting that nickel uptake is a limiting factor in urease biogenesis at a neutral pH. Under acidic conditions, when expression of the operon is optimal, the accumulation of intracellular Ni$^{2+}$ via the activity of UreMQO appears to be sufficient for the activation of all translated urease subunits, and additional nickel had no impact on the levels of urease activity. Utilizing the capacity of nickel accumulation as a limiting factor for total urease activity at neutral pH provides an additional level of control for urease expression in $S$. salivarius.

Upregulation of urease activity by NiCl$_2$ was regulated at the level of enzyme activation. The expression of urease genes and urease activity in $H$. pylori is regulated by the availability of nickel (27) and supplementation the growth medium with micromolar levels of NiCl$_2$ leads to higher levels of transcription of the operon. To investigate whether the upregulation of urease activity by NiCl$_2$ in $S$. salivarius could be mediated at the level of urease gene transcription from $p_{ureI}$, the level of ex-
pression was monitored in the recombinant *S. salivarius* strain *PureIcat*, which carries a single copy of the *pureI-cat* fusion at the *lacZ* locus (7), by measuring chloramphenicol acetyltransferase (CAT) specific activity. No significant differences in CAT activity were observed in cells grown in different concentrations of NiCl₂, regardless of the growth pH (data not shown), confirming that higher levels of urease activity in the presence of NiCl₂ did not result from elevated levels of ure transcription.

To further confirm that urease gene transcription was not regulated by NiCl₂ in *S. salivarius*, total cellular RNA was isolated from wild-type cells and the UreMQO-deficient strain grown in BHI-KPB that was supplemented with 0 to 100 μM NiCl₂ and the amounts of ureC- and ureM-specific mRNA were quantitated by slot blot analysis. No significant difference in the levels of ureC-specific mRNA could be detected in the wild-type strain under all concentrations of NiCl₂ tested (Fig. 7). Likewise, no differences in ureC-specific mRNA were detected between the wild-type and UreMQO-deficient strains in response to nickel concentrations, indicating that the nickel-responsive activation of urease activity is not mediated at the transcriptional level. As expected, the levels of ureM-specific message in the wild-type strain were not influenced by the amount of NiCl₂ in the growth medium (Fig. 7), and there was no detectable ureM signal in the UreMQO-deficient strain (data not shown).

To determine whether posttranscriptional regulation is a factor in the enhancement of urease activity by exogenous NiCl₂, we also examined the levels of UreC protein, the α subunit of the urease enzyme, by Western blot analysis with an anti-UreC polyclonal antibody in strains grown in different concentrations of NiCl₂. No significant differences in the levels of UreC were noted in the wild-type or UreMQO-deficient strains in response to the amount of NiCl₂ added to the medium (data not shown). These results indicate that the absence

**FIG. 6.** Urease specific activities in *S. salivarius* 57.1 and the UreMQO-deficient strain growing in BHI-KPB, BHI, and BHI-HCl containing 0 to 100 μM NiCl₂. The specific activities are expressed as nanomoles of urea hydrolyzed per minute per milligram of protein. The values shown are averages from multiple reaction sets from three independent samples. For each value reported, separate reaction sets with various times and cell quantities, which fell within the linear ranges of the standard curve, were used. All reactions were performed in triplicate. N.D., not detectable.

**FIG. 7.** Slot blot analysis of ureC- and ureM-specific mRNA. Total cellular RNA of wild-type 57.1 and UreMQO-deficient strains was isolated as described previously (9) and further purified by using the RNeasy total RNA kit (Qiagen). Panels: I, 10 μg of total RNA probed with a ureC-specific probe; II, 20 μg of total RNA probed with a ureM-specific probe. Columns 1 to 8 represent RNA isolated from cells grown in BHI-KPB supplemented with 0, 2.5, 5, 10, 25, 50, 75, and 100 μM NiCl₂, respectively. Negative controls are 10 μg of total cellular RNA treated with RNase A prior to loading.
of detectable urease activity in the UreMQO-deficient strains in the absence of exogenous NiCl₂ was due to the inability to transport NiCl₂ in the mutant rather than the lack of production of urease subunits and, again, that NiCl₂ does not regulate urease expression at the level of transcription or translation.

Summary. In conclusion, S. salivarius possesses a multicomponent nickel transporter belonging to the ABC transporter superfamily. In contrast to most pathogenic microorganisms, which generally possess more than one nickel uptake system, UreMQO is likely to be the only nickel-specific uptake system in S. salivarius. Nevertheless, other metal transport systems, presumably magnesium transporters, could also transport nickel when this metal was supplied in excess.

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