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Genetic and Physiologic Analysis of the *groE* Operon and Role of the HrcA Repressor in Stress Gene Regulation and Acid Tolerance in *Streptococcus mutans*

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Our working hypothesis is that the major molecular chaperones DnaK and GroE play central roles in the ability of oral bacteria to cope with the rapid and frequent stresses encountered in oral biofilms, such as acidification and nutrient limitation. Previously, our laboratory partially characterized the *dnaK* operon of *Streptococcus mutans* (*hrcA-grpE-dnaK*) and demonstrated that *dnaK* is up-regulated in response to acid shock and sustained acidification (G. C. Jayaraman, J. E. Penders, and R. A. Burne, Mol. Microbiol. 25:329–341, 1997). Here, we show that the *groESL* genes of *S. mutans* constitute an operon that is expressed from a stress-inducible σ^A-type promoter located immediately upstream of a CIRCE element. GroEL protein and mRNA levels were elevated in cells exposed to a variety of stresses, including acid shock. A nonpolar insertion into *hrcA* was created and used to demonstrate that HrcA negatively regulates the expression of the *groEL* and *dnaK* operons. The SM11 mutant, which had constitutively high levels of GroESL and roughly 50% of the DnaK protein found in the wild-type strain, was more sensitive to acid killing and could not lower the pH as effectively as the parent. The acid-sensitive phenotype of SM11 was, at least in part, attributable to lower F_1F_0-ATPase activity. A minimum of 10 proteins, in addition to GroES-EL, were found to be up-regulated in SM11. The data clearly indicate that HrcA plays a key role in the regulation of chaperone expression in *S. mutans* and that changes in the levels of the chaperones profoundly influence acid tolerance.

The ability to adapt to changes in environmental conditions is essential for the survival of microorganisms. Bacteria in oral biofilms are constantly subjected to acid shock and sustained acidification as a result of the rapid accumulation of acids generated from glycolysis by the acidogenic oral microflora. Central to the tolerance of environmental insults by microorganisms is the production of a variety of stress proteins, including the molecular chaperones GroEL and DnaK (12, 26). These proteins perform essential roles in cellular metabolism by assisting in the folding of newly synthesized or denatured proteins, as well as in the assembly, transport, and degradation of cellular proteins (9).

In bacteria, transcriptional regulators and alternative σ factors have been shown to be components of complex regulatory pathways that tightly control the transcription of heat shock genes under various conditions (28). In *Escherichia coli*, the primary mechanism for induction of the general stress response involves the modulation by DnaK of the activity of the alternative σ factor RpoH (σ^{32}), which controls transcription of a variety of stress genes, including *groEL* and *dnaK* (8, 15). In the gram-positive soil bacterium *Bacillus subtilis*, four classes of stress-regulated genes have been identified (14, 20). Class I genes, encoding GroEL and DnaK, constitute the CIRCE regulon and are negatively controlled by HrcA (37, 40). Class II genes are controlled by the alternative σ factor σ^B, and class III genes were recently shown to be negatively regulated by CtsR (14). Finally, class IV stress genes comprise those genes that are not regulated by one of the systems described above.

Control of class I stress gene expression in *B. subtilis* is governed by the widespread HrcA-CIRCE system. HrcA, a repressor protein, negatively regulates transcription of class I stress genes by binding to a DNA element called CIRCE (for controlling inverted repeat of chaperone expression) located in the regulatory regions of stress genes (20, 40). It also has been found that GroEL modulates the activity of the HrcA repressor (27), stabilizing and preventing aggregation of HrcA, which allows HrcA to function.

Streptococcus mutans, a bacterial pathogen associated with human dental caries, is well adapted to tolerate rapid changes in dental plaque pH, as well as fluctuations in carbohydrate availability and multiple environmental stresses. The ability of this organism to survive large and rapid changes in its environment, and in particular to grow and metabolize carbohydrates at low pH, is considered to be an important virulence factor. *S. mutans* is inherently more acid resistant than many other oral bacteria and is able to mount an acid tolerance response (ATR). The ATR is characterized by increased acid resistance, enhanced glycolytic capacities, and increased activity of the proton-translocating enzyme F_1F_0-ATPase (5, 18, 35). The ATR has also been found to confer cross-protection against multiple environmental insults, including UV radiation and oxidative stress (34). The basis for the ATR and cross-protection has been partially defined by the demonstration that acid-adapted cells have higher ATPase activities (5), as well as the

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identification of a low-pH-inducible DNA repair pathway (17) and an H^+-glucose symporter that operates at pH 5.0 (13). Additionally, several proteins are induced in response to growth at low pH, although the roles of these induced gene products in acid tolerance have not been evaluated (19, 39). Finally, separate studies have shown that two genes, *ffh* (16) and *sgp* (2), are required for acid tolerance. In spite of progress made on acid tolerance in oral streptococci, many of the gene products necessary for the inherent acid resistance of *S. mutans* and for the induction and phenotypic manifestation of the ATR by oral streptococci are yet to be defined.

The production of stress proteins, including the molecular chaperones GroEL and DnaK, is a central feature of bacterial stress responses (12). Unlike for *E. coli* and *B. subtilis*, there is very little information on modulation of stress gene expression in lactic acid bacteria. The first detailed description of the transcriptional organization and regulation of a stress response in lactic acid bacteria was for the *dnaK* operon of *S. mutans*, containing the *hrcA*, *grpE*, and *dnaK* genes, which are transcribed from a σ^A-type promoter (22). Additionally, by using a continuous chemostat culture, it was demonstrated that levels of *dnaK* mRNA and DnaK were increased in response to acid shock and remained elevated in acid-adapted cells (i.e., cells that had induced the ATR), suggesting that DnaK is intimately involved in responses to environmental acidification.

In this report, we identified and characterized the *groE* operon of *S. mutans* (*groES-groEL*) and explored the regulation of *groEL* expression in response to various stresses, including heat, acidic pH, ethanol, and H_2O_2. To evaluate the role of HrcA as a repressor protein in stress gene regulation, an HrcA-deficient strain was constructed, and the effects of inactivation of *hrcA* on *groE* and *dnaK* expression were assessed. The mutant, which produced elevated levels of GroEL and diminished levels of DnaK, was used to establish a molecular link between chaperone gene expression and acid tolerance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains DH10B and M15 were grown in Luria broth, and *S. mutans* UA159 was grown in brain heart infusion broth (BHI). When needed, kanamycin (40 μg ml^{-1} for *E. coli* or 500 μg ml^{-1} for *S. mutans*) or ampicillin (100 μg ml^{-1}) was added to the medium. To investigate the response of *S. mutans* to different stress conditions, cells were grown in BHI at 37°C to mid-exponential phase (optical density at 600 nm [OD_{600}] ≅ 0.6), and aliquots of the cultures were then heat shocked at 42°C or incubated in the presence of ethanol (20 mM) or H_2O_2 (2 mM) for different periods of time. For studies involving acid shock and acid adaptation, *S. mutans* was grown in a continuous chemostat culture as previously described (22).

DNA manipulations. Chromosomal DNA was prepared from *S. mutans* as previously described (10). Plasmid DNA was isolated from *E. coli* by using QIAgen columns (Qiagen, Chatsworth, Calif.), and restriction and DNA-modifying enzymes were obtained from Life Technologies, Inc. (LTI; Gaithersburg, Md.), New England Biolabs (Beverly, Mass.), or MBI Fermentas (Amherst, N.Y.). PCRs were carried out with 100 ng of *S. mutans* chromosomal DNA by using *Vent* DNA polymerase, and PCR products were purified with the QIAquick kit (Qiagen). DNA was introduced into *S. mutans* by natural transformation (33) and into *E. coli* by electroporation (36). Southern blot analyses were carried out at high stringency as described by Sambrook et al. (36).

Overexpression and purification of HrcA and antibody production. The *hrcA* gene was amplified from *S. mutans* UA159 by PCR with primers containing restriction sites (*Bam*HI and *Pst*I) to facilitate cloning into the plasmid expression vector pQE30 (Qiagen). The resulting plasmid (pJL1), which produced a recombinant protein with an in-frame fusion of six consecutive histidine residues to the N terminus of HrcA, was used to transform *E. coli* M15. His-tagged HrcA was purified from isopropyl-β-D-thiogalactopyranoside (IPTG)-treated cells under denaturing conditions by following the protocols recommended by the supplier (Qiagen). The recombinant protein (0.4 mg) was further purified by excision from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and used to elicit a polyclonal antiserum in rabbits (Lampire, Pipersville, Pa).

Construction of an *hrcA* insertion mutant. A 2.1-kb fragment containing the *hrcA* gene was amplified by PCR and cloned onto pGEM-5Zf(+) (Promega, Madison, Wis.) to generate plasmid pJL2. The *hrcA* gene was then inactivated by replacing a region containing the σ^A-type promoter located upstream of *hrcA* (P1) and a 5′ portion of the structural gene with a kanamycin resistance gene (ΩKm element) that is flanked by strong transcription and translation terminators (32). To allow for expression of genes downstream of *hrcA* in the *dnaK* operon, which appear to be essential for cell viability (see Results), the *Streptococcus salivarius* urease promoter (P*ureI*) (11) was also cloned immediately downstream of the insertion site of ΩKm into the *hrcA* gene. Specifically, a 0.31-kb fragment of *hrcA* in pJL2 was replaced by a 3.3-kb ΩKm-P*ureI* fragment to give plasmid pJL6. Circular plasmid pJL6 was used to transform *S. mutans*, and transformants were selected on BHI agar with kanamycin. Chromosomal DNAs from the mutant and wild-type strains were digested with *Bgl*II and probed with the *hrcA* gene and the ΩKm element. The absence of HrcA in the recombinant strains was further confirmed by Western blot analysis. One such isolate, designated SM11, was used throughout the studies.

Primer extension and RNA analysis. Total RNA was extracted from cells of *S. mutans* UA159 and the *hrcA* mutant strain (SM11) growing in chemostat cultures or from mid-exponential-phase batch cultures by using a protocol described by Chen et al. (11). Primer extensions were carried out with the oligonucleotide designated EXTGROE (5′-CTAGCACCAGCAAGGAC-3′) by a protocol described previously (1) with primer annealing and reverse transcription performed at 42°C. For quantitative slot blot analysis, equivalent amounts of denatured RNA were transferred to nylon membranes (GeneScreen Plus; NEN Life Science products, Inc., Boston, Mass.) by a slot blot apparatus (LTI) as described by Sambrook et al. (36). For Northern blot analyses, total RNA was separated on a 1.0% formaldehyde gel following the protocol described elsewhere (1). RNAs were UV cross-linked to the membranes, and filter membranes were probed with *S. mutans groEL* or *dnaK* probes labeled with [α-^{32}P]dATP (NEN Life Science). All hybridizations and washes were carried out under high-stringency conditions. Signals obtained on autoradiographs were quantified with an IS1000 digital imaging system (Alpha Innotech Corp, San Leandro, Calif.). Reverse transcriptase PCR (RT-PCR) was performed with the ThermoScript RT-PCR System from LTI.

Protein electrophoresis and Western blotting. Cells grown as described above were centrifuged, washed, and homogenized in the presence of glass beads by using a Bead Beater (Biospec, Bartlesville, Okla.), as described previously (11). Whole-cell lysates were separated by SDS-PAGE, blotted onto Immobilon-P membranes (Millipore, Bedford, Mass.), and subjected to Western blot analysis by standard techniques (1). Two-dimensional (2-D) electrophoresis was performed according to the method of O'Farrell (29) at Kendrick Labs, Inc. (Madison, Wis.). Isoeletrofocusing was carried out with 2.0% pH 4 to 8 ampholines (Gallard-Schlesinger, Garden City, N.Y.), and 2-D electrophoresis was carried out in a SDS–10% polyacrylamide gel. Gels were stained with Coomassie blue and dried between sheets of cellophane.

pH drop and acid killing. The glycolytic capacities of the HrcA-deficient (SM11) and wild-type (UA159) strains were compared by pH drop experiments according to the protocol described by Belli and Marquis (5). Briefly, cells from steady-state chemostat cultures of UA159 or SM11 were harvested, washed with 1 culture volume of cold distilled water, and resuspended in a solution of 50 mM KCl and 1 mM $MgCl_2$ in 1/10 of the original culture volume. The suspension was titrated with 0.1 M KOH to a pH of 7.2, and pH drops were initiated by addition of 55.6 mM glucose. In order to determine the ability of SM11 to resist acid killing, steady-state cultures of the wild-type and mutant strains grown under the conditions described above were washed once with 1% peptone (pH 7.0) and resuspended in 1/10 of the original volume in 1% peptone (pH 3.0). Samples were stirred continuously at room temperature, aliquots of cells were removed at predetermined intervals, and the viable counts of each culture were determined by plating on BHI agar.

F_1F_0-ATPase assays. The F_1F_0-ATPase activity of permeabilized cells prepared from chemostat-grown cells was determined according to the method of Belli and Marquis (5). Briefly, cells were collected, washed once with membrane buffer (75 mM Tris [pH 7.0], 10 mM $MgSO_4$), and concentrated 25-fold in the same buffer. Cells were then permeabilized in the presence of toluene, collected by centrifugation, and resuspended in the same volume of membrane buffer. Permeabilized samples were mixed with 50 mM Tris-maleate buffer (pH 6.0)

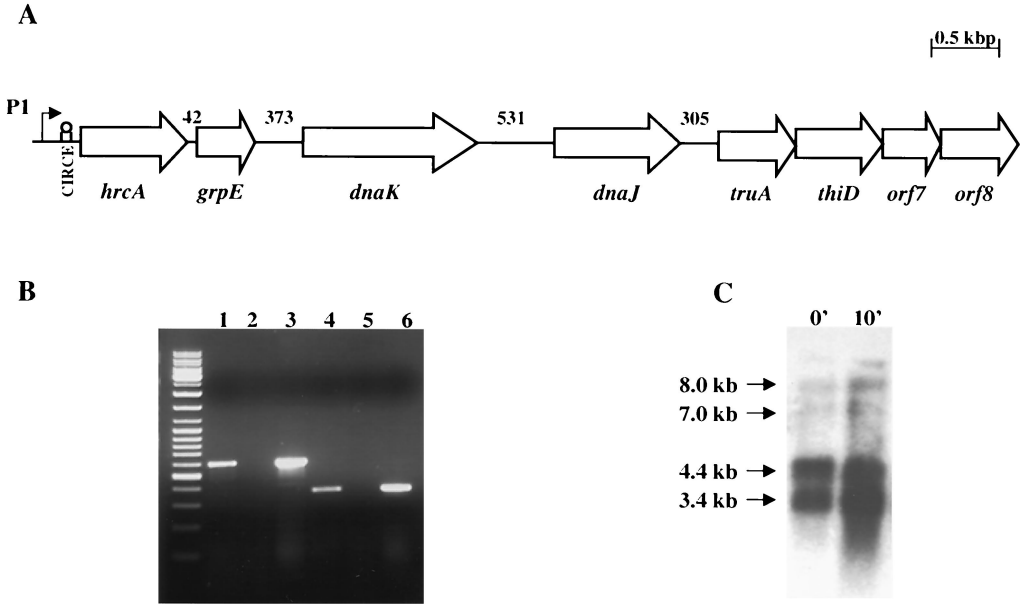

FIG. 1. (A) Transcriptional organization of the *S. mutans dnaK* operon. (A) Schematic diagram of the *S. mutans dnaK* operon and genes downstream of the operon. Upstream of *hrcA* is the *fruAB* operon involved in the utilization of extracellular polysaccharides (22). *truA* is predicted to encode a tRNA-modifying enzyme and *thiD* phosphomethyl pyrimidine kinase, which is possibly involved in thiamine biosynthesis. The numbers above the schematic indicate the size of the intergenic regions in base pairs. (B) PCR products generated by RT-PCR. Shown are ethidium bromide-stained 1% agarose gels of products obtained with primers spanning the intergenic regions between *dnaK-dnaJ* (lanes 1 to 3) and *dnaJ-truA* (lanes 4 to 6). Products from the PCR were derived with the following: cDNA prepared from *S. mutans* RNA (lanes 1 and 4); a negative control using *S. mutans* RNA, but omitting RT (lanes 2 and 5); and *S. mutans* chromosomal DNA (lanes 3 and 6). A DNA ladder mix from Fermentas was used as the molecular weight markers. (C) Northern blot analysis of *S. mutans* UA159 *dnaK*. Cells were grown at 37°C to mid-exponential phase and then subjected to heat shock at 42°C. RNA was isolated from cells before (0′) and after (10′) heat shock for 10 min. Total RNA (10 μg per lane) was separated in a 1.0% gel, transferred to a nylon membrane, and hybridized to a *dnaK*-specific probe.

containing 10 mM $MgSO_4$, and the reactions were initiated by adding 0.5 M ATP (pH 6.0) to a final concentration of 5 mM at 37°C. Samples were removed at 0, 15, 30, and 45 min and assayed for inorganic phosphate released from ATP with the Fiske-Subbarow reagent (Sigma, St. Louis, Mo).

RESULTS

Genetic organization of the *dnaK* operon. Previous studies in our laboratory indicated that the *dnaK* operon of *S. mutans* contained the *hrcA*, *grpE*, and *dnaK* genes. BLAST search analysis of databases maintained as part of the *S. mutans* Genome Sequencing Project at the University of Oklahoma's Advanced Center for Genome Technology identified the *dnaK* operon (starting at position 83510) and revealed the presence of an open reading frame (ORF4) of 1,131 bp located 3′ to *dnaK* and starting 531 bp from the *dnaK* stop codon (Fig. 1A). The deduced amino acid sequence of ORF4 had a predicted molecular mass of 40.8 kDa and showed a high degree of similarity (up to 83%) to DnaJ proteins of other bacteria. Analysis of the region 3′ to *dnaJ* revealed an ORF (ORF5) of 747 bp starting 305 bp from the *dnaJ* stop codon. ORF5 was predicted to encode a 249-amino-acid polypeptide with a predicted molecular mass of 28 kDa that exhibited high levels of similarity (86%) to a putative tRNA pseudouridine synthase A (TruA) protein from *Streptococcus pyogenes*. Immediately downstream of *truA* is an ORF similar to that coding for phosphomethylpyrimidine kinases, which are generally involved in thiamine biosynthesis, followed by two ORFs with the highest degree of similarity to those coding for hypothetical proteins of *S. pyogenes*. None of the ORFs 3′ to *dnaJ* share homology to genes that have been proven to be transcribed as part of eubacterial *dnaK* operons, nor do they share homology with proteins required for stress tolerance.

The use of RT-PCR revealed evidence for a transcript containing the *dnaK-dnaJ* sequences, indicating that *dnaJ* is probably cotranscribed with *hrcA, grpE*, and *dnaK* from the σ^A-type promoter (P1) (22) located 5′ to *hrcA* (Fig. 1B). Surprisingly, with a set of primers containing internal sequences of the *dnaJ* and *truA* genes, a band corresponding to the correct molecular size was observed, suggesting that transcription of the *dnaK* operon may proceed beyond the *dnaJ* gene (Fig. 1B). Northern analysis (Fig. 1C), revealed the presence of two major mRNA species migrating at 3.4 and 4.4 kb, consistent with the sizes of the *dnaKJ* and *grpE-dnaKJ* transcripts, and these transcripts were increased in cells that had undergone heat shock for 10 min. These mRNAs represented over 99% of the total *dnaK*-containing mRNA as assessed by densitometry. Of note, the two major transcripts found to hybridize with a *dnaK* probe were present in Northern blots as revealed by a *dnaJ* probe (data not shown), further confirming the RT-PCR results that showed that *dnaJ* is cotranscribed with *dnaK*. Also consistent with the RT-PCR results, it was found that a small fraction (<1%) of the total mRNA hybridizing to the *dnaK* and *dnaJ* probes was present as transcripts of approximately 7.0 and 8.0 kb (Fig. 1C). The most reasonable interpretation of these data is that termination of the highly expressed *dnaK* operon is not 100% efficient, and in some cases, the downstream genes can be cotranscribed as part of the operon.

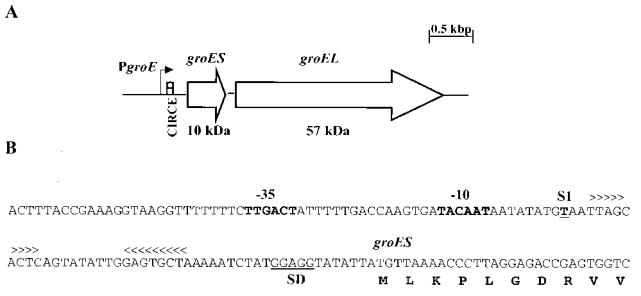

FIG. 2. (A) Schematic diagram of the *groE* operon of *S. mutans*. (B) Sequence of the P*groE* region. The σ^A-type −35 and −10 regions are in boldface. A transcriptional initiation site, S1, mapped by primer extension, is shown in boldface and underlined. The CIRCE element is marked with arrowheads. The predicted Shine-Dalgarno (SD) sequence is underlined.

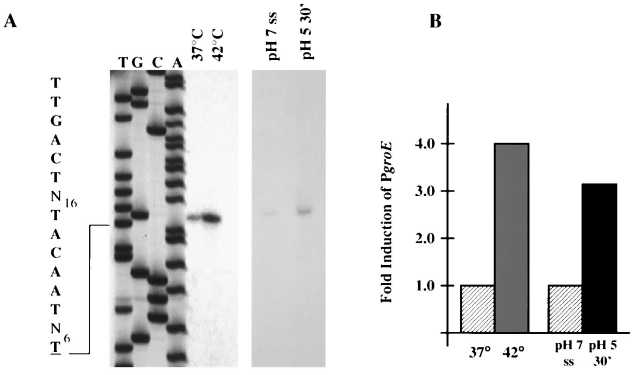

FIG. 3. Mapping of the *S. mutans groE* promoter (P*groE*) by primer extension. (A) Total RNA was isolated from mid-log-phase cultures grown at 37°C or 30 min after heat shock at 42°C or from chemostat-grown cells that have reached steady state at pH 7.0 (pH 7 ss) or 30 min after acid shock at pH 5.0. Adjacent to the primer extension is a sequencing reaction performed with plasmid pJL14 with the same primer used in the primer extension reaction (EXTGROE). Indicated on the left are the sequences of the −35 and −10 regions of the σ^A-type promoter (boldface) and the corresponding transcriptional initiation site (boldface and underlined). Images of primer extension products under each stress condition were obtained from different exposures of X-ray film with a digital imaging system. (B) Bar graph showing P*groE* induction as determined by densitometry with an IS1000 digital imaging system.

Genetic organization and transcriptional mapping of the *groE* operon. The *groES* and *groEL* genes from *S. mutans* were identified in the *S. mutans* genome by BLAST searches (Fig. 2). The *groE* operon is located from position 1834692 through position 1832649 and is transcribed in the opposite orientation to the *dnaK* operon. The *groES* gene (285 bp) encoded a polypeptide of 95 amino acids with a predicted molecular mass of 10 kDa. The *groEL* gene (1,626 bp) encoded a 542-amino-acid protein with a predicted molecular mass of 57 kDa. As expected, the proteins encoded by *groES* and *groEL* showed high levels of similarity to known GroES and GroEL proteins found in other gram-positive bacteria, including *S. pyogenes* (71% GroES and 91% GroEL), *Streptococcus pneumoniae* (81% GroES and 89% GroEL), and *Streptococcus agalactiae* (80% GroES and 90% GroEL). To locate the transcriptional initiation site(s) of the *groE* operon and to determine whether transcription was induced in response to heat shock and acidic conditions, primer extensions were performed with total RNA isolated from mid-log cultures subjected to heat shock or from chemostat cultures growing at steady state at pH 7.0 that had been subjected to an acid shock at pH 5.0. A single transcription initiation site was identified 48 nucleotides 5′ to the translational initiation site. A σ^A-type promoter (TTGACT-N_{16}-TACAAT), located 57 bp 5′ to the *groES* start codon, was identified. Densitometric analysis of this single band demonstrated that transcription from this promoter, designated P*groE*, was increased fourfold in heat-shocked cells and threefold in acid-shocked cells (Fig. 3). A perfect CIRCE element (TTAGCACTC-N_9-GAGTGCTAA) was identified starting 11 bp 3′ to the −10 element and 19 bp from the *groES* start codon. Northern analyses with a fragment of the *groEL* gene as a probe revealed the presence of a single transcript of 2.1 kb, indicating that *groEL* can be cotranscribed with *groES* from P*groE* (Fig. 4).

Induction of *groEL* mRNA and GroEL protein in response to environmental stresses. To determine whether *groEL* expression is modulated by environmental conditions, slot blots of total RNA and Western blot analyses were performed. Steady-state continuous culture or batch cultures of UA159 grown under the conditions described in Materials and Methods were submitted to different stresses. Slot blot analysis revealed that the levels of *groEL* mRNA were induced approximately 2.5-fold when pH 7.0 steady-state cultures were submitted to acid shock (pH 5.0) for up to 1 h. In contrast to what was previously observed for *dnaK* (22), no significant differences in the *groEL* mRNA levels in steady-state cells grown at either pH 7.0 or 5.0 were observed (Fig. 5A and B). The results of Western blot analyses also indicated that the levels of *S. mutans* GroEL were elevated following acid shock, but were not altered in steady-state pH 5.0 cells compared with steady-state pH 7.0 cells (Fig. 5C and D). As seen with the induction of *dnaK* mRNA and DnaK (22), the magnitude of

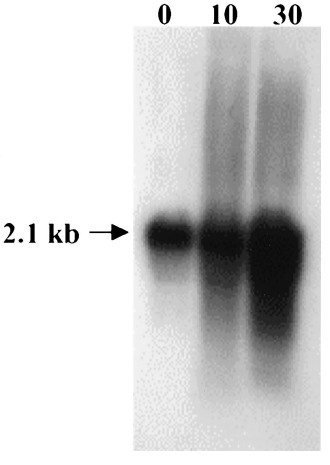

FIG. 4. Northern blot analysis of *S. mutans* UA159 *groEL*. Cells were grown at 37°C to the mid-log phase and then subjected to heat shock at 42°C. RNA was isolated from cells before (time zero) and after heat shock for 10 and 30 min. Total RNA (10 μg per lane) was separated in a 1.0% gel, blotted to nylon membrane, and hybridized to a *groEL*-specific probe.

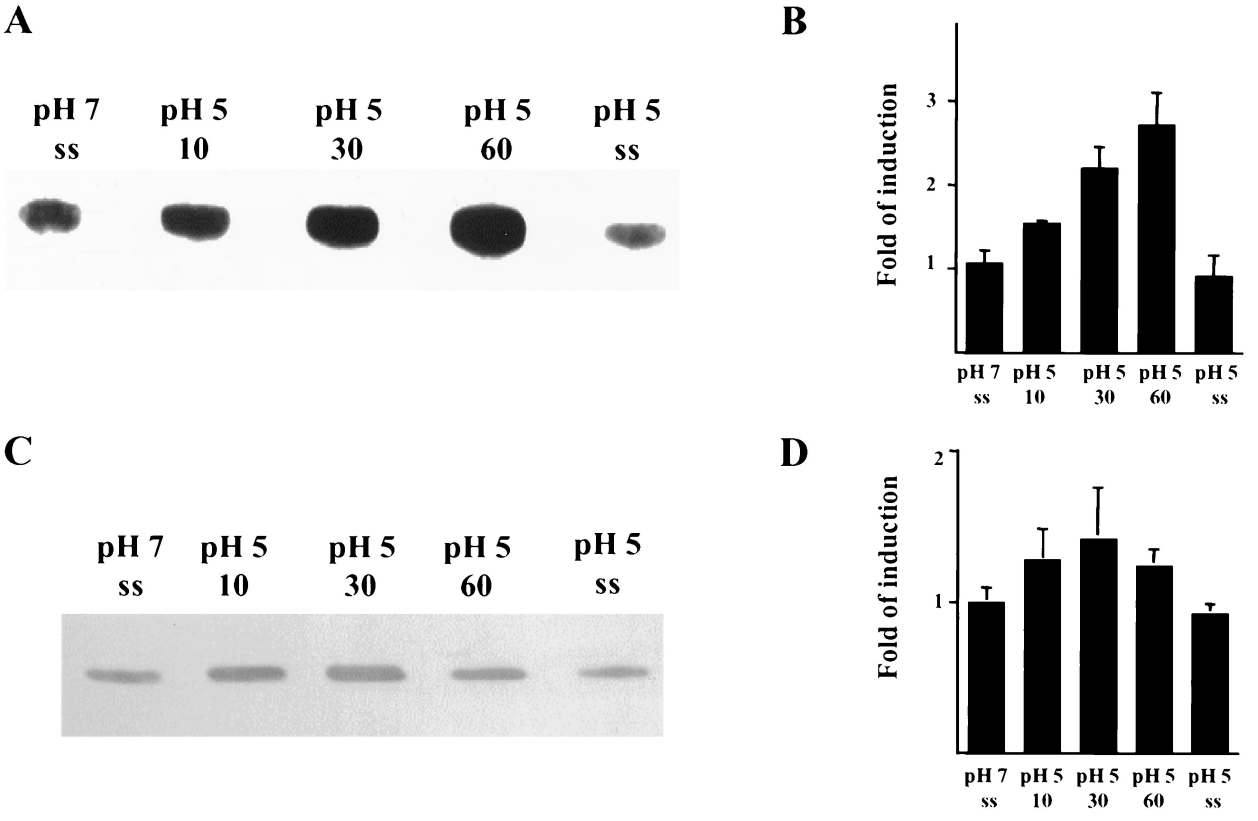

FIG. 5. Induction of *groEL* mRNA and GroEL in reponse to changes in environmental pH. (A) Slot blot of total RNA isolated from samples grown in the chemostat under the conditions indicated in the figure (ss, steady-state). RNase-treated samples were used as controls (data not shown). Hybridization was performed with an internal fragment of *groEL*. (B) Bar graph depicting induction of *groEL* mRNA as measured by densitometry. The graph shows the averages and standard deviations of three independent experiments. (C) Western blot analysis of GroEL levels with a polyclonal antibody against *S. pyogenes* GroEL (1:1,000). (D) Bar graph showing induction of GroEL as measured by densitometry.

the increase in *groEL* RNA is much greater than for the gene product. This is most likely attributable to the fact that both DnaK and GroEL are highly abundant proteins and that large increases in the amount of mRNA are needed to effect significant changes in the absolute quantity of these proteins in the cell. Although acid is believed to be the most prominent stress factor with which *S. mutans* is confronted, organisms in dental biofilms are subjected to many other types of stresses. In addition to acid shock, it was found that the levels of *groEL* mRNA and GroEL were elevated when *S. mutans* was subjected to heat shock, oxidative stress, and exposure to ethanol (Fig. 6).

Inactivation of the *S. mutans hrcA* gene leads to constitutive expression of *groES* and *groEL*. To determine the role of HrcA in stress gene regulation of *S. mutans*, the *hrcA* gene was disrupted by double crossover insertion of a kanamycin resistance gene flanked by strong transcription and translation terminators (ΩKm) (32) and followed by the *S. salivarius* urease

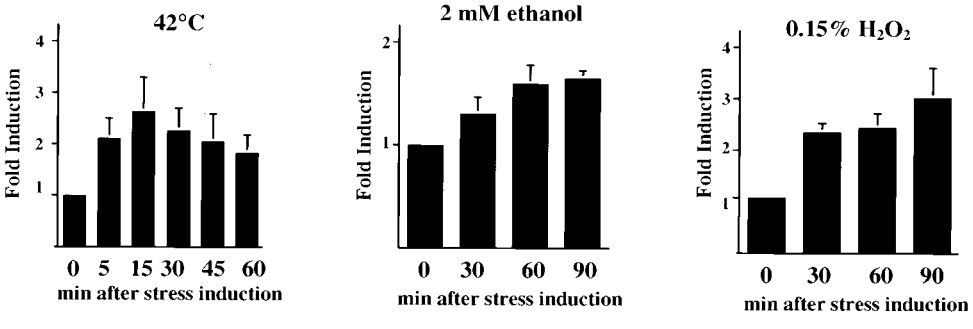

FIG. 6. Quantitative slot blot analysis of *groEL* mRNA in reponse to environmental stresses. Total RNA was isolated from mid-log-phase cultures that were submitted to the conditions indicated in the figure. The calculated mRNA induction profile of each stress condition was determined by densitometry ($n = 2$).

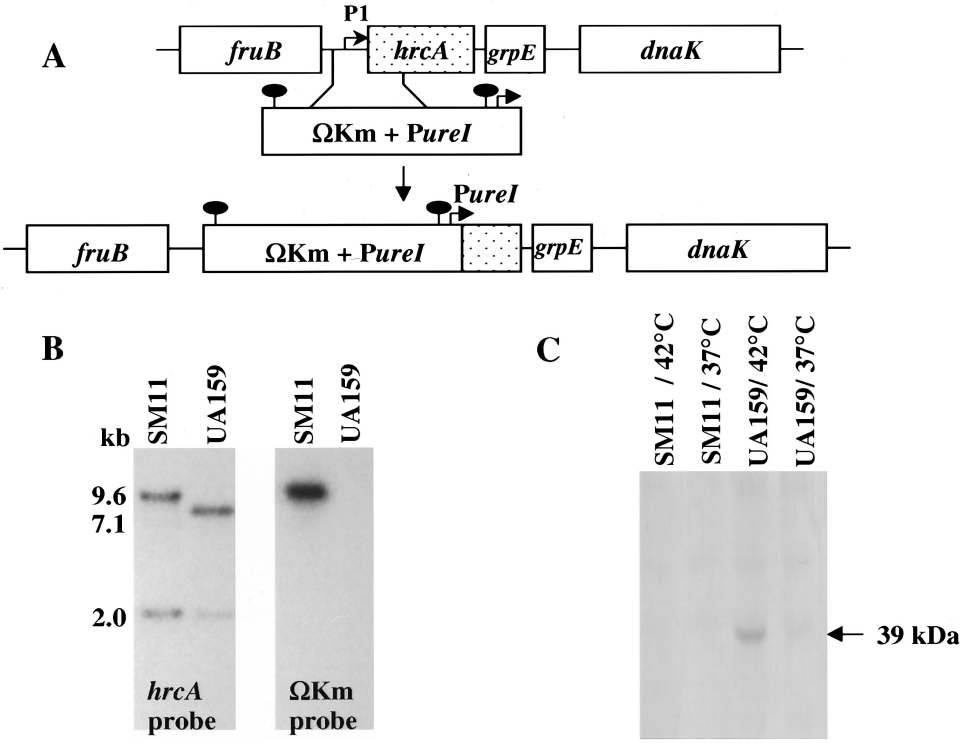

FIG. 7. Construction of an *hrcA* mutant strain (SM11). (A) Schematic diagram of the insertional inactivation of the *hrcA* gene in *S. mutans* UA159. The gene was inactivated by replacing a region containing the σ^A-type promoter (P1) upstream of *hrcA* and a 5′ portion of the structural gene by a cassete containing the ΩKm element and the P*ureI* promoter. The solid oval markings indicate the transcriptional and translational terminators of the ΩKm element. (B) Southern hybridization to *S. mutans* chromosomal DNA by using the *hrcA* gene and the ΩKm element as probes. Indicated are the sizes of hybridizing bands as determined by comparison with DNA standards. (C) Western blot analysis of HrcA protein in response to heat shock (42°C). Total protein lysates of UA159 and SM11 were obtained under denaturing conditions and probed with anti-*S. mutans* HrcA antibody (diluted 1:500).

promoter (P*ureI*), (11) (Fig. 7). Initially, attempts to inactivate *hrcA* by inserting the strongly polar ΩKm cassette alone resulted in isolation of only single crossover insertions. Similar results were obtained when attempts were made to isolate strains with insertions of ΩKm into the *dnaK* gene (data not shown). By using P*ureI* to drive the expression of the genes 3′ to *hrcA* in the *dnaK* operon, a strain with a double crossover insertion into the *hrcA* gene was then isolable. Correct integration of ΩKm was confirmed by Southern blotting and by Western blotting with an anti-*S. mutans* HrcA antibody (Fig. 7). It is interesting that, as previously observed at the mRNA level (22), the levels of HrcA protein were elevated after heat shock (Fig. 7C), consistent with the fact that *hrcA* is transcribed from a stress-inducible promoter. Also, similar to other organisms, HrcA proved to be highly insoluble. The protein could only be purified from recombinant *E. coli* strains and was only detectable in *S. mutans* when the cell lysates were obtained under denaturing conditions.

Primer extension analysis with RNA from wild-type and mutant strains demonstrated that inactivation of *hrcA* led to derepression of P*groE* under both homeostatic and acidic conditions, with 5- and 3.4-fold inductions, respectively, when compared to the wild-type strain growing under the same conditions (Fig. 8A). It was not possible to obtain primer extension data from the σ^A-type promoter (P1) of the *dnaK* operon in strain SM11, because the *hrcA* mutant was constructed such that P1 and the 5′ portion of *hrcA* were replaced by the ΩKm/P*ureI* cassette. Instead, the expression from P1 in an *hrcA* mutant strain was analyzed by primer extension in a strain in which the *hrcA* locus was inactivated by single crossover insertion of a suicide plasmid (HRCERM) (22). The results indicated that HrcA also represses transcription of P1 in the *dnaK* operon (data not shown).

The levels of *groEL* and *dnaK* mRNA in the mutant and wild-type strains were compared under different stress conditions. Under acid stress and all other conditions tested, the levels of *groEL* mRNA were significantly elevated in the mutant strain, demonstrating that HrcA represses *groEL* transcription (Fig. 8B). Similar results were obtained when blots were probed with *groES* (data not shown). Diminished levels of *dnaK* mRNA were observed (Fig. 8B), probably because transcription of *dnaK* from P*ureI* was not as efficient or the mRNA was not as stable as when transcription arises from the cognate *dnaK* promoter (P1). Western blot analysis with anti-*S. pyogenes* GroEL and DnaK polyclonal antibodies (25) also confirmed the results obtained by RNA analysis indicating that GroEL was constitutively derepressed in the *hrcA* mutant strain, whereas the levels of DnaK were diminished relative to UA159 (data not shown).

A 2-D PAGE approach was used to assess differences in protein expression of exponential phase cells of SM11 and UA159. Strong induction of the GroEL (4.2-fold) and GroES

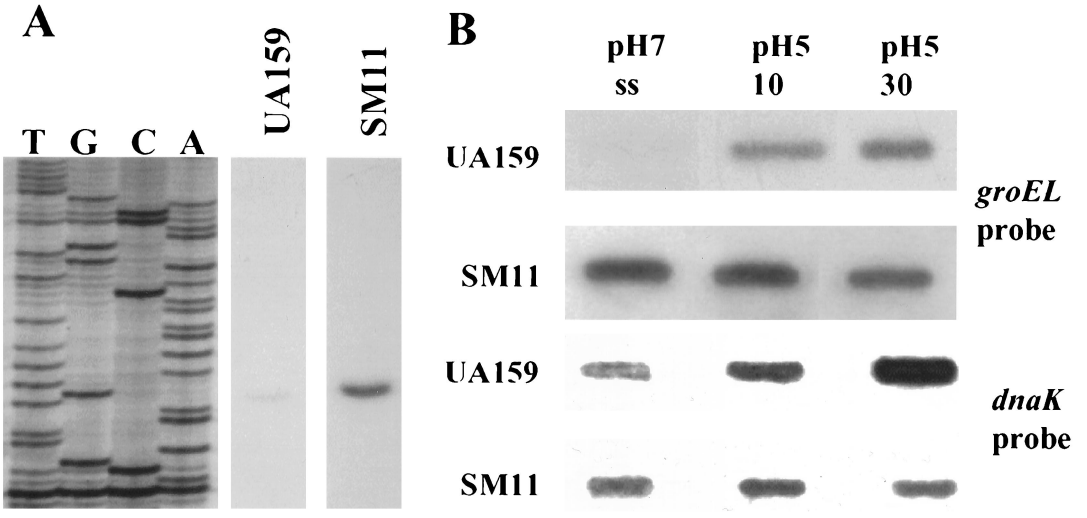

FIG. 8. Primer extension and slot blot analysis of an *hrcA* mutant strain (SM11). (A) Total RNA was isolated from pH 7.0, steady-state, chemostat-grown cells of *S. mutans* UA159 or SM11. Equal amounts of RNA were hybridized to the primer EXTGROE (Fig. 2) and subjected to primer extension analysis. Images of primer extension products from UA159 and SM11 RNAs were obtained from the same piece of X-ray film with a digital imaging system. Intervening lanes were omitted to present the relevant data. (B) Slot blot of total RNA from UA159 and SM11 probed with internal fragments of the *groEL* or *dnaK* genes.

(4.5-fold) proteins was observed in SM11, whereas DnaK expression was approximately 45% lower in SM11 (Fig. 9), consistent with the Western blot data. Interestingly, in addition to alterations in molecular chaperone expression, at least 10 other proteins appeared to be up-regulated in the mutant strain.

Acid tolerance of the *hrcA* mutant strain. At 37°C, SM11 had a generation time of about 70 min, slower than the 50-min doubling time of the wild-type strain. To determine if SM11 had altered resistance to low pH, acid killing and pH drop experiments were performed with cells obtained from steady-state continuous culture. Steady-state pH 7.0 (unadapted) or pH 5.0 (acid-adapted) cultures of SM11 and UA159 were subjected to acidification at pH 3.0. Regardless of the growth conditions, the mutant strain was killed more rapidly at pH 3.0 than the wild-type strain growing under the same conditions (Fig. 10A), indicating an acid-sensitive phenotype for SM11. Consistent with the acid-sensitive phenotype, pH drop experiments revealed that UA159 was consistently able to lower the pH through glycolysis to values lower than that which could be achieved by strain SM11 (Fig. 10B and Table 1). In spite of the overall acid-sensitive phenotype and changes in the final pH values achieved, acid-adapted SM11 cells (grown at pH 5) were able to reduce the pH to values lower than those of the unadapted SM11 cells (grown at pH 7) and became more resistant to acid killing, indicating that the *hrcA* mutant strain is able to mount an ATR.

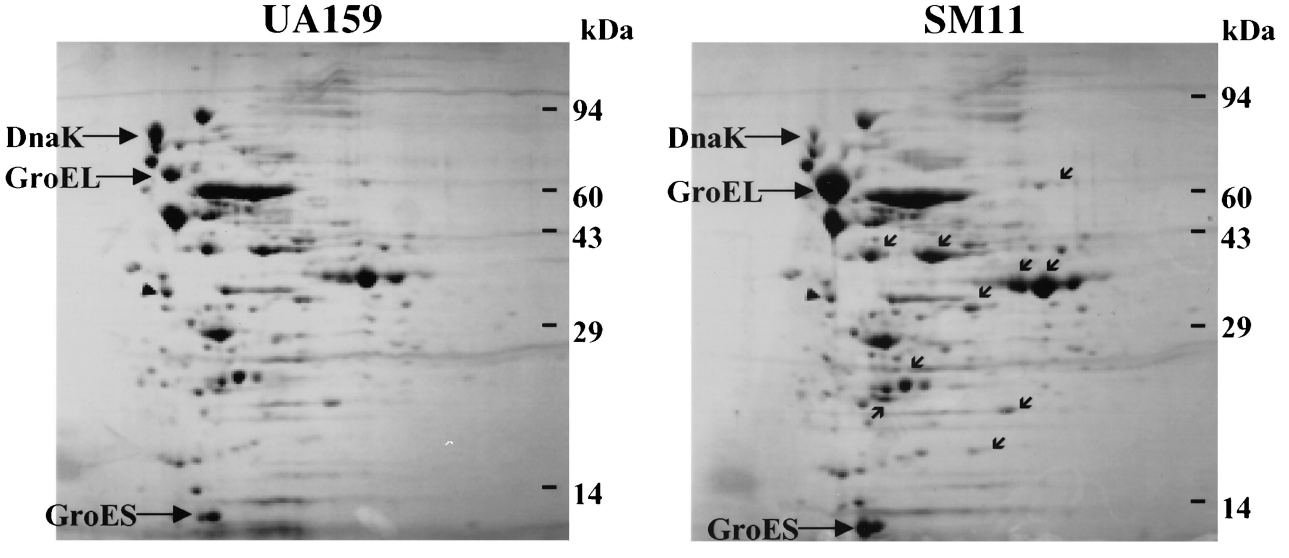

FIG. 9. 2-D protein pattern of *S. mutans* strains UA159 and SM11. Samples were grown in BHI and harvested in the exponential growth phase ($OD_{600} \cong 0.6$). GroEL, GroES, and DnaK are identified. Small arrows indicate other protein spots with increased expression in the SM11 strain.

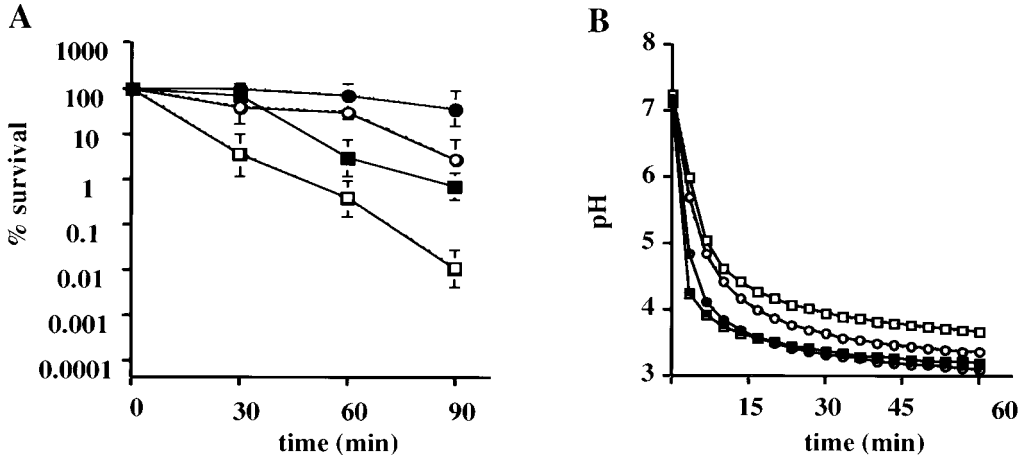

FIG. 10. Acid tolerance response of *S. mutans* UA159 (solid symbols) and SM11 (open symbols). Cells were collected from a steady-state chemostat held at pH 7.0 (squares) or pH 5.0 (circles) and subjected to acid killing or pH drop experiments. (A) Survival of UA159 and SM11 during acid challenge at pH 3.0. Cell viability at each time point is expressed as the percentage of viable cells (CFU per milliliter of culture) at time zero. (B) Glycolytic pH profile of UA159 and SM11. Glucose was added to the cell suspensions to initiate glycolysis, and pH drops were continuously monitored for 1 h. Data points were collected every 10 s, and data for every 2 min and 20 s are presented. The graph shows the averages and standard deviations of three independent experiments.

It has been demonstrated previously that acid adaptation of oral streptococci is correlated with an increase in proton-extruding ATPase activity (5). The F_1F_0-ATPase activity of both UA159 and SM11 was elevated in acid-adapted cells when compared to that in unadapted cells (4.2- and 2.7-fold increases, respectively). When comparing the ATPase activity of the two strains, the levels obtained from unadapted cells were very similar for UA159 and SM11. However, acid-adapted cells of SM11 showed substantially lower levels of ATPase than acid-adapted cells of UA159 (Table 1).

DISCUSSION

The *dnaK* operon (*hrcA-grpE-dnaK*) has been partially characterized in *S. mutans* (22). In the present study, we identified an ORF with high homology to *dnaJ* that was located 531 bp 3′ to *dnaK*. Frequently, in other gram-positive bacteria, *dnaK* operons consist of *hrcA*, *grpE*, and *dnaKJ* as well as other genes (4, 21, 30). Our results clearly indicate that the *S. mutans dnaK* operon consists of at least four genes: *hrcA*, *grpE*, and *dnaKJ.*

TABLE 1. Acid-adaptive response of *S. mutans* UA159 and SM11

Strain	Growth pH	1-h glycolytic pH (final pH)[a]	ATPase activity (nmol of PO_4/mg [dry wt])[b]
UA159	7.0	3.303 (±0.08)	0.0578 (±0.0062)
	5.0	3.208 (±0.111)	0.2429 (±0.0658)
SM11	7.0	3.562 (±0.107)	0.0689 (±0.0103)
	5.0	3.33 (±0.075)	0.1862 (±0.062)

[a] Differences in the final pH reached from pH 7-grown cells were statistically significant ($P < 0.05$). Although the differences in the final glycolytic pH values achieved by pH 5-grown cells of UA159 and SM11 were not statistically significant, because of day-to-day variation in the final pH values achieved by a given strain (about 0.1 pH unit), in every instance, the mutant was never able to lower the pH as low as the wild-type strain.

[b] ATPase activity is expressed as the amount of PO_4 liberated 30 min after the addition of ATP. The results presented here are the means and standard deviations of three independent experiments.

In *B. subtilis*, it has been demonstrated that the *dnaK* operon is heptacistronic (*hrcA grpE dnaK dnaJ orf35 orf28 orf50*) (21). A putative protein showing strong homology to the *B. subtilis* ORF35 protein (63% similarity) was found 3′ to *dnaJ* in *Clostridium acetobutylicum* and *Staphylococcus aureus* (4, 30). However, BLAST searches against the *S. mutans* genome did not indicate the presence of an ORF35 homologue near the *dnaK* locus. Instead, there is an ORF 3′ to *dnaJ* that has high levels of similarity to a putative tRNA pseudouridine synthase A gene (*truA*) from *S. pyogenes*, followed by a gene that may participate in pyrimidine metabolism or thiamine biosynthesis, and then by two genes with products of unknown function. Although we were able to detect transcriptional readthrough from the *dnaK* operon into *truA* by using the very sensitive RT-PCR technique, we question the biological significance of this finding, based on the fact that the genes downstream of *dnaJ* seem unlikely to participate in stress tolerance, and the overwhelming majority of the transcripts we observed by Northern blotting terminate at *dnaJ.* Thus, we believe that the *dnaK* operon is a four-gene operon that terminates at *dnaJ*, and any transcription into the *truA* gene probably arises because the terminator 3′ to *dnaJ* is not 100% efficient.

It is also noteworthy that the organization of the *S. mutans dnaK* operon is unique when compared with the same operon of other eubacteria and gram-positive cocci. In particular, there are two unusually large intergenic regions between *grpE* and *dnaK* and *dnaK* and *dnaJ* in the *dnaK* operon of *S. mutans*. Using reporter gene fusions and primer extension analysis, no functional promoter was found within the 373-bp *grpE-dnaK* intergenic region (22). In *B subtilis*, *dnaJ* is transcribed from a σ^A-dependent promoter preceding the entire *dnaK* operon, as well as from a vegetative promoter located downstream of *dnaK* (21). Preliminary results obtained by primer extension analysis and reporter gene fusions have not revealed a functional promoter in the *dnaKJ* intergenic region of *S. mutans* (data not shown). Thus, there do not appear to be internal

promoters driving *dnaJ* expression in the *S. mutans dnaK* operon, and transcription of *dnaJ* is probably dependent on P1. We propose that the intergenic regions play a role in posttranscriptional regulation of chaperone expression, perhaps in the processing or stability of the *dnaKJ* mRNAs. Along these lines, the Northern blots indicate that *hrcA* may not be a part of the most abundant transcripts, which most likely contain *grpE-dnaKJ* and *dnaKJ*. Although this is consistent with the fact that the DnaK chaperone complex is far more abundant than HrcA, it appears that mRNA processing may be an important component of regulation of expression of the genes in the *dnaK* operon, as has been shown in *B. subtilis* (21).

A previous report from our laboratory (22) and the present study demonstrated that *groEL* and *dnaK* are part of the general stress response of *S. mutans* and that the *hcrA* gene product is a major component controlling *dnaK* and *groE* expression. We are primarily interested in the role of the DnaK and GroE chaperone machines in acid tolerance and regulation of adaptation to acidic conditions. In particular, it has been demonstrated that the glycolytic activity of oral bacteria in biofilms on the tooth and other surfaces of the mouth can lower the pH from 7 to 4 in just a few minutes (23). Bacteria present in the biofilms on teeth must be able to withstand these rapid and substantial fluctuations in the environmental pH. Previously, it was shown that rapid acidification of steady-state cultures of *S. mutans* growing at neutral pH resulted in increases in the levels of *dnaK* mRNA and DnaK protein, and the levels remained elevated (approximately 1.8-fold) in steady-state cultures grown at pH 5.0 (22). As was observed for *dnaK*, the levels of *groEL* mRNA and GroEL were elevated during acid shock. However, after cells had achieved steady state at pH 5.0, which is known to result in full induction of the ATR, the levels of *groEL* mRNA and GroEL protein were indistinguishable from those seen in steady-state cultures at pH 7.0. Thus, both GroEL and DnaK are induced during the acid shock response, whereas acid adaptation involves maintenance of elevated levels of DnaK, but not of GroEL.

Despite the fact that DnaK is important for survival under extreme conditions, the role of this protein in acid tolerance and acid adaptation has not been defined. In order to evaluate the role of *S. mutans* DnaK in stress responses, we have tried to construct a DnaK-deficient strain by inserting a variety of different antibiotic resistance cassettes in the 5′ portion of the *dnaK* gene. Taking into consideration that a *dnaK* mutant strain would probably exhibit a temperature- and acid-sensitive phenotype, in addition to using standard conditions to isolate a *dnaK* mutant strain (e.g., nonbuffered BHI and incubation at 37°C), we used buffered media and a lower temperature (25°C) to try to allow growth of putative mutants. However, all attempts to produce a *dnaK* mutant failed. Also, as indicated above, we were unable to use the ΩKm element to isolate an *hrcA* mutant, likely due to the polar effects of this insertion on the expression of the downstream genes. These results indicate that, in contrast to *E. coli* and *B. subtilis*, a *dnaK* mutant of *S. mutans* may not be isolable, at least under the conditions tested. Similar findings have been reported for *Streptomyces coelicolor*, in which a *dnaK* mutation was apparently lethal (7). Such observations clearly point to a central role of the DnaK protein in physiologic homeostasis in *S. mutans*.

The presence of a gene with high homology to *hrcA* in the *dnaK* operon and the identification of the CIRCE element in the promoter regions of the *S. mutans groE* and *dnaK* operons suggested that HrcA could negatively regulate these two operons. To circumvent problems of DnaK deficiency, yet still be able to investigate the role of HrcA, an *hrcA* mutant strain (SM11) was isolated by inserting the ΩKm fragment containing an outward-reading promoter (P*ureI*), such that the genes downstream of *hrcA* could still be expressed. The data obtained here strongly indicated that the *groE* and *dnaK* operons of *S. mutans* were negatively controlled by HrcA in a manner similar to that of class I heat shock genes in *B. subtilis*. Although there was about a 50% reduction of *dnaK* expression in the *hrcA* mutant strain, the cells were viable and grew normally, albeit more slowly than the wild-type strain.

Compared to the parental strain, *S. mutans* SM11 was more sensitive to acid killing and showed a reduced capacity to produce acid at low pH values. The F_1F_0-ATPase is essential for pH homeostasis in *S. mutans*, and it is thought that an increase in the activity of this enzyme is an important contributor to the mounting of the ATR by this organism (35). Consistent with these ideas, acid-adapted cells of SM11 showed diminished levels of F_1F_0-ATPase compared to those in UA159 cells, whereas unadapted cells from both strains had comparable levels of the enzyme. This suggests that under conditions of acid stress, *S. mutans* is unable to produce sufficient ATPase or cannot maintain the integrity of the ATPase in the face of cytoplasmic or membrane acidification. The basis for lower ATPase activity is not entirely clear at this point, but it is well established that the DnaK chaperone and its cochaperones, GrpE and DnaJ, play an important role in essential cellular processes, including assisting in the folding of nascent or denatured proteins, as well as in translocation of proteins. Therefore, it is possible that the DnaK-DnaJ-GrpE complex is involved in the biogenesis of the ATPase complex or in stabilizing this complex at low pH. It is reasonable to speculate that under normal conditions, the reduced amount of DnaK that is present in SM11 is still sufficient to assist F_1F_0-ATPase folding, assembly, or membrane insertion, explaining why comparable levels of ATPase activity were observed in both the wild-type and mutant strain growing at pH 7.0. Under acid stress conditions, there is an apparent need for increased ATPase activity to maintain the internal pH near neutrality, but DnaK may be titrated by denatured proteins, resulting in insufficient levels of DnaK to chaperone the ATPase complex. Interestingly, overexpression of the GroESL chaperonin was not sufficient to compensate for the decrease in DnaK in terms of biogenesis of the F-ATPase. In many cases, the GroE chaperonin seems to be more important in processing oligomeric complexes than the DnaK machinery. However, it has been suggested that the folding of proteins larger than 60 to 70 kDa, as is the case for the ATPase complex, cannot be properly assisted by GroESL (9). From the data obtained here, it can be hypothesized that DnaK and not GroESL is responsible for assisting the biogenesis of the ATPase complex. If this hypothesis is correct, involvement of DnaK with F-ATPases could be at the level of deaggregation, facilitation of proper folding and assembly, or participation of DnaK in insertion of the F_0 portion into the membrane. The concept of an interaction between DnaK and F-ATPase is supported by studies that have demonstrated that depletion of hsp70, a eukaryotic DnaK homologue, affected

the translocation of the β subunit of the F_1F_0-ATPase of *Saccharomyces cerevisae* (3).

It is also important to note that the acid-sensitive phenotype of SM11 could be unrelated to a direct effect of changes in the levels of DnaK or GroESL. It has been reported that GroEL positively modulates the HrcA repressor activity (27). One possibility is that GroEL regulates other stress response repressors as it does HrcA. In this case, the mutant strain, which had high levels of GroEL, could display a stress-sensitive phenotype as a result of a failure to derepress other pathways needed for stress tolerance. In fact, the *hrcA* mutant strain does have enhanced sensitivity to killing by heat (50°C) and H_2O_2 (0.15%) (data not shown). Additionally, 10 other protein spots were elevated in the mutant strain. Whether HrcA negatively regulates these proteins or if the altered expression of these polypeptides occurs as a consequence of changes in expression of GroEL or DnaK is not known. However, after searching the available sequence of the *S. mutans* genome database, we have found no evidence of the presence of a CIRCE element in regions that are not linked to the *groE* and *dnaK* operons, suggesting that HrcA may not directly impact genes other than those in the *dnaK* and *groE* operons.

Other studies have demonstrated that altered expression of GroEL and DnaK can lead to enhanced susceptibility to stresses and reduced cell viability (6, 24, 31, 38). For example, in *E. coli* and *Haemophilus ducreyi*, the overexpression of DnaK and DnaJ, which are known to negatively regulate the stress response in these organisms, leads to decreased expression of GroEL and GroES, resulting in diminished cell survival (6, 31). In *Lactococcus lactis*, a C-terminal deletion of DnaK resulted in increased expression of all CIRCE-containing heat shock operons, including the *dnaK* operon itself, and led to a pronounced temperature-sensitive phenotype (24).

The results presented here support that the repressor of class I heat shock gene expression, HrcA, and the major molecular chaperones of *S. mutans* play central roles in growth and stress tolerance by these important pathogens. In addition, we have shown that DnaK may play a key role in acid tolerance, perhaps by participating in the biogenesis or stabilization of the ATPase complex. Studies are under way to dissect the molecular genetics and biochemistry of the genes in the *dnaK* and *groE* operons in tolerance of environmental acidification.

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