NOTES

The F-ATPase Operon Promoter of *Streptococcus mutans* Is Transcriptionally Regulated in Response to External pH

Wendi L. Kuhnert,^{1†} Guolu Zheng,² Roberta C. Faustoferri,²
and Robert G. Quivey, Jr.^{1,2*}

Center for Oral Biology, Aab Institute for Biomedical Sciences,² and Department of Microbiology and Immunology,¹ University of Rochester, Rochester, New York

Received 25 May 2004/Accepted 15 September 2004

***Streptococcus mutans* F-ATPase, the major component of the acid-adaptive response of the organism, is transcriptionally upregulated at low pH. Fusions of the F-ATPase promoter to chloramphenicol acetyltransferase indicated that pH-dependent expression is still observed with a short promoter that contains a domain conserved between streptococcal ATPase operons.**

During growth at acidic pH values, *Streptococcus mutans* and *S. sanguis* both increase production of their F-ATPases (4, 5, 12). By pumping protons out of cells, the enzymes function to assist in maintaining internal pH values (6, 8, 13, 14). The ATPases from *S. mutans* and *S. sanguis* differ in their innate abilities to function in acidic conditions, as evidenced by the pH optima of the enzymes: approximately 6.0 for *S. mutans* and 7.0 for *S. sanguis*, with the maximal induction of the *S. mutans* enzyme occurring at pH 5. Thus, the ATPase is likely a significant reason why the mutans streptococci are able to outcompete *S. sanguis* during growth in low-pH environments (21) and to cause dental caries (18, 24).

The present study was undertaken to determine whether control of ATPase production in oral streptococci occurs at the transcriptional level. RNA slot blots, reporter gene fusions, and computer modeling were used to examine the pH-dependent expression of the *S. mutans* F-ATPase promoter in *S. mutans* and *S. sanguis*.

(The material presented here was used in partial fulfillment of the Ph.D. requirements of the University of Rochester by W. L. Kuhnert.).

RNA samples from cells grown at pH 5 have greater abundance of ATPase β -subunit transcript. We first determined whether ATPase-specific mRNA levels differed in RNA samples prepared from cells grown at pH 5 or 7. *S. mutans* UA159 was grown in continuous culture to steady state at a dilution rate of 0.24 h⁻¹, as described previously (10, 15). RNA slot blots hybridized to a β -subunit-specific DNA probe (amplified with primers β Fwd and β Rev; Table 1) showed an approximately twofold increase in the abundance of ATPase message in the cells grown at pH 5 (Fig. 1).

ATPase operon transcriptional rate is greater at pH 5 than at pH 7. Three reporter gene fusions were constructed using PCR (25), both in multicopy (plasmid-borne) and single-copy (chromosomal) fusions, to determine the transcriptional activity of the operon. These included (i) an intact promoter fragment of the *S. mutans* GS-5 ATPase operon fused to a *cat* gene (contained on pDL556/CAT and in *S. mutans* UR123); (ii) a fragment containing the -10 and -35 sequences and promoter sequence that extended only into the predicted inverted DNA repeat proximal to the structural gene (carried on pDL680/CAT and in *S. mutans* UR124); and (iii) a promoterless *cat* gene fusion (contained on pDLCAT and in *S. mutans* UR122). The appropriate clones of promoter fragments were verified by nucleotide sequencing (15, 26) and were subsequently subcloned into pDL278 (16, 17), resulting in the constructs listed above (detailed in Fig. 2).

Single-copy fusions were created by integrating the promoter-chloramphenicol acetyltransferase (CAT) fusions (described above) into the *amyA* locus (1) of the chromosome and by using plasmid pBGK (31) as a transformation control. Genotypes of recombinant strains UR122, UR123, and UR124 (detailed above) were confirmed by PCR.

Initially, CAT assays were performed on protein extracts of the plasmid-borne *cat* fusion-bearing strains grown in batch culture to obtain data regarding transcription of the *atp* operon during growth in the absence of pH control. CAT activity (27) and protein concentrations (7) were determined as described previously. As expected, the streptococcal strains containing the promoterless control plasmid, pDLCAT, showed no CAT activity (Table 2). Expression of the fusions in *S. mutans* UA159 showed that the shorter construct (pDL680/CAT) resulted in less CAT activity than the full-length fusion (pDL556/CAT) (Table 2). The decreased levels of CAT activity seen with the shorter fusion suggested that the entire promoter region (including both inverted repeat regions) played a role in the expression of the *atp* operon in both *S. mutans* and *S. sanguis*.

The identical strains were also grown in the chemostat under

* Corresponding author. Mailing address: Center for Oral Biology, Box 611, 601 Elmwood Ave., Rochester, NY 14642. Phone: (585) 275-0382. Fax: (585) 276-0190. E-mail: Robert_Quivey@urmc.rochester.edu.

† Present address: Division of Viral Hepatitis, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333.

TABLE 1. Strains and plasmids

Strain, oligonucleotide, or plasmid	Source, reference, or sequence ^a	Relevant feature
Strains		
<i>S. mutans</i> GS5	11	<i>atp</i> promoter
<i>S. mutans</i> UA159	1, 22	Test strain
<i>S. sanguis</i> 10904	American Type Culture Collection	Test strain
<i>S. mutans</i> UR122	This study	Single-copy fusion
<i>S. mutans</i> UR123	This study	Single-copy fusion
<i>S. mutans</i> UR124	This study	Single-copy fusion
<i>E. coli</i> DH10B	Invitrogen	Cloning host
<i>E. coli</i> DH5 α	Invitrogen	Cloning host
<i>E. coli</i> GBE180	23	Cloning host
Oligonucleotides		
3' CAT-BAM	5' CAAGGATCCAAGCTTCGACGAATT 3'	Amplify <i>cat</i> gene
BGL II CAT	5' GCGAAGGAAAGATCTATGGAGAAAAAATCAC 3'	Amplify <i>cat</i> gene
BGL II ATP	5' ATTCAACATAGATCTTTCTCCTTC 3'	Amplify <i>atp</i> promoter
BRL P556BAM	5' CGACGGATCCTCTCTTTTGGACCTAATA 3'	Amplify <i>atp</i> promoter
BRL P680BAM	5' CGACGGATCCTTTCTTGTTCATTGAC 3'	Amplify <i>atp</i> promoter
AMY-HIN	5' GGCCAAGCTTCAAACGATGGTAAGCATTGG 3'	Amplify <i>amyA</i>
AMY-ECO	5' CCGGAATTCCGTTTGCAATTAGCTTCCC 3'	Amplify <i>amyA</i>
β Fwd.	5' TTCAGTTGCCAAGGTTGG 3'	Amplify <i>atpD</i>
β Rev.	5' AAAGGTGGTAAAGTCGGC 3'	Amplify <i>atpD</i>
Plasmids		
pGEM-T	Promega	
pUC:CAT	R. A. Burne (University of Florida)	
pGEM-7Zf+	Promega	
pDL278	16, 17	
pDL556/CAT	This study	Full-length <i>atp_P</i> -CAT fusion
pDL680/CAT	This study	Short-length <i>atp_P</i> -CAT fusion
pDLCAT	This study	Promoterless CAT fusion
pUC-amy	This study	<i>amyA</i> clone
pUC-amy556	This study	Full-length <i>atp_P</i> -CAT fusion in <i>amyA</i>
pUC-amy680	This study	Short-length <i>atp_P</i> -CAT fusion in <i>amyA</i>
pUC-amyCAT	This study	Promoterless CAT fusion in <i>amyA</i>
pBGK	31	<i>S. mutans</i> integration vector

^a Restriction sites are denoted by the underlined sequence. Double-underlined sequence denotes the ATG start site of the *atp* operon.

pH-controlled conditions. The strains were grown first at pH 7.0, under glucose-limiting conditions, and then the pH was lowered to pH 5.0 or 6.0 for *S. mutans* and *S. sanguis*, respectively. CAT activity data from *S. mutans* showed that the longer fusion constructs again expressed greater CAT activity compared to that of the shorter fusion, though the differences were somewhat less pronounced (Table 3). These observations were in agreement with the data obtained from the batch-grown cells.

Following the experiments with multicopy, plasmid-borne reporter fusions, we examined the activities of the strains bearing single-copy constructs. Results were similar to those seen with the episomal strains (Table 4).

Overall, the data indicated that the full-length fusion was likely to be necessary for optimal expression of the *atp* promoter. Nevertheless, the effects of extracellular pH on ATPase transcription were still manifested in the short fusion. This observation supports the notion that pH-mediated regulation may be exerted near the structural genes, possibly by an alternative sigma factor acting at a core region containing the -35 and -10 sequences, as postulated for *S. pneumoniae* (20). The transcriptional activity of the fusions of the *S. mutans* ATPase promoter in the *S. sanguis* background indicates the likelihood that regulation of the ATPase is similar in both organisms.

Slot blot analysis of ATPase mRNA in plasmid-borne, fu-

sion-bearing strains. To ensure that ATPase message levels correlated with CAT activity in the fusion-bearing strains, experiments were conducted to estimate the effect of pH on the level of F-ATPase-specific mRNA. Total RNA, prepared from cells harvested from chemostat cultures of *S. mutans* and *S. sanguis* strains containing the fusion plasmids, was treated prior to blotting as previously described (26). Treated samples were serially diluted into aliquots as indicated in Fig. 1, and they were applied to duplicate membranes. The first blot of each pair was probed with a 16S rRNA-encoding DNA probe, as previously described (28), to establish a baseline for RNA

FIG. 1. Slot blots of RNA prepared from *S. mutans* UA159 grown at steady state at pH 5 or 7. Total cellular RNA samples were serially diluted as shown and were probed with the β -subunit-specific probe.

TABLE 4. Single-copy *S. mutans atp* promoter-fusion CAT activity in steady-state cultures of *S. mutans*^a

Strain	CAT activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
<i>S. mutans</i> UR122, pH 7	0.0 $\pm$ 0.0
<i>S. mutans</i> UR122, pH 5	0.0 $\pm$ 0.0
<i>S. mutans</i> UR123, pH 7	0.7 $\pm$ 0.0 a,b
<i>S. mutans</i> UR123, pH 5	1.0 $\pm$ 0.0 a,c
<i>S. mutans</i> UR124, pH 7	0.4 $\pm$ 0.0 d,b
<i>S. mutans</i> UR124, pH 5	0.7 $\pm$ 0.1 d,e

^a Data represents assays performed in triplicate for each extract. Letters indicate statistically different values, between pairs, using two-tailed Student's *t* test with $P \leq 0.05$ and 95% confidence interval.

5) such that ATPase-encoding RNA levels were higher in cells grown at a pH value of 6.0 than in the cells grown at pH 7.0.

Control experiments, estimating the copy number of the plasmid-borne fusions, showed that differences between strains were not statistically significant (data not shown).

ATPase promoter in *S. mutans* contains repeated patterns.

Our observations indicated the possibility of two types of domains in the *atp* promoter that were conserved between *S. mutans* and *S. sanguis*. Subsequent motif searches (2, 3) were conducted, with 6, 10, 15, 25, or 50 bp as the maximum window size and 3 or 6 bp as the minimum window size. The analyses resulted in the prediction of three motifs with significant probabilities in streptococcal *atp* operon promoters. Motif 1, 5'-TAGGACTAGACTAAGCAAGCGCA-3', and motif 2, 5'-AAAGTTGTGAAGGGGAATTCA-3', were the more distributed patterns, each occurring more than once in the promoter sequences. The third motif, 5'-AAAATTTTGAAGGA GAATCC-3', was located immediately upstream of the coding region of the operon, inside the transcriptional start site at -29 (Fig. 2).

A model for the regulation of the operon could include two or three types of sites for a regulatory protein(s): one for full transcriptional activity of the operon, and a second domain, closer to the structural genes, which may confer pH sensitivity for transcriptional upregulation. Such a model of regulation could then be described as a single protein interacting at all of

TABLE 5. RNA transcript levels of *atpD* in steady-state cultures of *S. mutans* UA159 and *S. sanguis* 10904 containing *S. mutans atp* promoter fusion plasmids

Strain	Phosphorimager units (10^{-7}) ^a
<i>S. mutans</i> UA159(pDL556/CAT), pH 7	1.2 $\pm$ 0.2 a
<i>S. mutans</i> UA159(pDL556/CAT), pH 5	3.2 $\pm$ 0.9 b
<i>S. mutans</i> UA159(pDL680/CAT), pH 7	0.9 $\pm$ 0.1 c,d
<i>S. mutans</i> UA159(pDL680/CAT), pH 5	1.7 $\pm$ 0.2 c,e
<i>S. mutans</i> UA159(pDL680/CAT), pH 7	1.3 $\pm$ 0.4 f
<i>S. mutans</i> UA159(pDL680/CAT), pH 5	3.6 $\pm$ 0.5 f
<i>S. sanguis</i> 10904(pDL556/CAT), pH 7	0.1 $\pm$ 0.1 a
<i>S. sanguis</i> 10904(pDL556/CAT), pH 6	0.3 $\pm$ 0.1 b
<i>S. sanguis</i> 10904(pDL680/CAT), pH 7	1.7 $\pm$ 0.0 d
<i>S. sanguis</i> 10904(pDL680/CAT), pH 6	2.2 $\pm$ 0.0 e
<i>S. sanguis</i> 10904(pDL680/CAT), pH 7	0.1 $\pm$ 0.0
<i>S. sanguis</i> 10904(pDL680/CAT), pH 6	0.3 $\pm$ 0.1

^a Units are presented for the RNA probed with *atpD* as normalized data obtained using a radiolabeled 16S rRNA probe. Letters indicate statistically different values, between pairs, using two-tailed Student's *t* test with $P \leq 0.05$ and 95% confidence interval.

the predicted sites but with differing affinities. The incremental increases in ATPase activity, observed with incremental decreases in extracellular pH (19), could be explained by the successive loss or gain of protein binding at the motif sites. Alternatively, multiple proteins could be involved with *atp* operon regulation, reflecting entirely different binding sites and roles for proteins binding at motifs 1, 2, and 3. The motifs predicted in this study must be considered preliminary models that will become refined as our understanding of streptococcal gene regulation improves. A similar approach recently identified motifs of approximately 40 bp in size for OxyR binding sites in *Escherichia coli* (32), including one such site that was *dsbG* proximal, that is, inside the -35 region.

We have demonstrated that the *S. mutans* ATPase operon is regulated by a mechanism that includes DNA elements located upstream of the putative -35 site. The regulatory scheme appears to include sensitivity to environmental pH, which also seems to be conserved in other streptococci, though *S. sanguis* is known to be less aciduric than *S. mutans* (30). The mechanism through which the potential regulatory sequences exert their effects remains to be determined.

We thank R. E. Marquis, R. A. Burne, and Y.-Y. M. Chen for helpful discussion throughout. We also thank Raymond C. Ogawa for assistance with the RNA experiments and E. Fozo for help with the statistical analysis.

The work was supported by grants from the NIH, National Institute for Dental and Craniofacial Research (DE-06127, DE-11549), and the Cariology Training Program (T32-DE07165) (W.L.K.).

REFERENCES

- Ajdic, D., W. M. McShan, R. E. McLaughlin, G. Savic, J. Chang, M. B. Carson, C. Primeaux, R. Tian, S. Kenton, H. Jia, S. Lin, Y. Qian, S. Li, H. Zhu, F. Najjar, H. Lai, J. White, B. A. Roe, and J. J. Ferretti. 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc. Natl. Acad. Sci. USA* **99**:14434-14439.
- Bailey, T. L., and C. Elkan. 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers, p. 28-36. *In* R. Altman, D. Brutlag, P. Karp, R. Lathrop, and D. Searls (ed.), Second International Conference on Intelligent Systems for Molecular Biology, vol. 1. AAAI Press, Menlo Park, Calif.
- Bailey, T. L., and M. Gribskov. 1998. Methods and statistics for combining motif match scores. *J. Comput. Biol.* **5**:211-221.
- Belli, W. A., and R. E. Marquis. 1991. Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. *Appl. Environ. Microbiol.* **57**:1134-1138.
- Belli, W. A., and R. E. Marquis. 1994. Catabolite modification of acid tolerance of *Streptococcus mutans* GS-5. *Oral Microbiol. Immun.* **9**:29-34.
- Bender, G. R., S. V. Sutton, and R. E. Marquis. 1986. Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci. *Infect. Immun.* **53**:331-338.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
- Dashper, S. G., and E. C. Reynolds. 1992. pH regulation by *Streptococcus mutans*. *J. Dent. Res.* **71**:1159-1165.
- Ferretti, J. J., W. M. McShan, D. Ajdic, D. J. Savic, G. Savic, K. Lyon, C. Primeaux, S. Sezate, A. N. Suvorov, S. Kenton, H. S. Lai, S. P. Lin, Y. Qian, H. G. Jia, F. Z. Najjar, Q. Ren, H. Zhu, L. Song, J. White, X. Yuan, S. W. Clifton, B. A. Roe, and R. McLaughlin. 2001. Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proc. Natl. Acad. Sci. USA* **98**:4658-4663.
- Fozo, E. M., and R. G. Quivey Jr. 2004. Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic conditions. *Appl. Environ. Microbiol.* **70**:929-936.
- Gibbons, R. J., K. S. Berman, P. Knoettner, and B. Kapsimalis. 1966. Dental caries and alveolar bone loss in gnotobiotic rats infected with capsule forming streptococci of human origin. *Arch. Oral Biol.* **11**:549-560.
- Hamilton, I. R., and N. D. Buckley. 1991. Adaptation by *Streptococcus mutans* to acid tolerance. *Oral Microbiol. Immun.* **6**:65-71.
- Kobayashi, H. 1985. A proton-translocating ATPase regulates pH of the bacterial cytoplasm. *J. Biol. Chem.* **260**:72-76.
- Kobayashi, H., T. Suzuki, and T. Unemoto. 1986. Streptococcal cytoplasmic

- pH is regulated by changes in amount and activity of a proton-translocating ATPase. *J. Biol. Chem.* **261**:627–630.
15. Kuhnert, W. L., and R. G. Quivey Jr. 2003. Genetic and biochemical characterization of the F-ATPase operon from *Streptococcus sanguis* 10904. *J. Bacteriol.* **185**:1525–1533.
  16. LeBlanc, D. J., and L. N. Lee. 1991. Replication function of pVA380-1, p. 235–239. In G. M. Dunne, P. P. Cleary, and L. L. McKay (ed.), *Genetics and molecular biology of streptococci and enterococci*. American Society for Microbiology, Washington, D.C.
  17. LeBlanc, D. J., L. N. Lee, A. Abu, and A. Jaibat. 1992. Molecular, genetic, and functional analysis of the basic replicon of pVA380-1, a plasmid of oral streptococcal origin. *Plasmid* **28**:130–145.
  18. Loesche, W. J. 1986. Role of *Streptococcus mutans* in human dental decay. *Microbiol. Rev.* **50**:353–380.
  19. Ma, Y., T. M. Curran, and R. E. Marquis. 1997. Rapid procedure for acid adaptation of oral lactic-acid bacteria and further characterization of the response. *Can. J. Microbiol.* **43**:143–148.
  20. Martin-Galiano, A. J., M. J. Ferrandiz, and A. G. de la Campa. 2001. The promoter of the operon encoding the F₀F₁ ATPase of *Streptococcus pneumoniae* is inducible by pH. *Mol. Microbiol.* **41**:1327–1338.
  21. McDermid, A. S., A. S. McKee, D. C. Ellwood, and P. D. Marsh. 1986. The effect of lowering the pH on the composition and metabolism of a community of nine oral bacteria grown in a chemostat. *J. Gen. Microbiol.* **132**:1205–1214.
  22. Murchison, H. H., J. F. Barrett, G. A. Cardineau, and R. Curtiss III. 1986. Transformation of *Streptococcus mutans* with chromosomal and shuttle plasmid (pYA629) DNAs. *Infect. Immun.* **54**:273–282.
  23. Pierais, V. L., and G. J. Barcak. 1999. Development of *E. coli* host strains tolerating unstable DNA sequences on ColE1 vectors. *Focus* **21**:18–19.
  24. Quivey, R. G., Jr., W. L. Kuhnert, and K. Hahn. 2000. Adaptation of oral streptococci to low pH. *Adv. Microb. Physiol.* **42**:239–274.
  25. Quivey, R. G., Jr., R. C. Faustoferri, W. A. Belli, and J. S. Flores. 1991. Polymerase chain reaction amplification, cloning, sequence determination and homologies of streptococcal ATPase-encoding DNAs. *Gene* **97**:63–68.
  26. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning. A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
  27. Shaw, W. V. 1979. Chloramphenicol acetyltransferase activity from chloramphenicol-resistant bacteria. *Methods Enzymol.* **43**:737–755.
  28. Slots, J., A. Ashimoto, M. J. Flynn, G. Li, and C. Chen. 1995. Detection of putative periodontal pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. *Clin. Infect. Dis.* **20**:S304–S307.
  29. Smith, A. J., R. G. Quivey, Jr., and R. C. Faustoferri. 1996. Cloning and nucleotide sequence analysis of the *Streptococcus mutans* membrane-bound, proton-translocating ATPase operon. *Gene* **183**:87–96.
  30. Sturr, M. G., and R. E. Marquis. 1992. Comparative acid tolerances and inhibitor sensitivities of isolated F-ATPases of oral lactic acid bacteria. *Appl. Environ. Microbiol.* **58**:2287–2291.
  31. Wen, Z. T., and R. A. Burne. 2001. Construction of a new integration vector for use in *Streptococcus mutans*. *Plasmid* **45**:31–36.
  32. Zheng, M., X. Wang, B. Doan, K. A. Lewis, T. D. Schneider, and G. Storz. 2001. Computation-directed identification of OxyR DNA binding sites in *Escherichia coli*. *J. Bacteriol.* **183**:4571–4579.