Journal of Bacteriology, May 1995, p. 2622–2727 Vol. 177, No. 10
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Sequence, Expression, and Function of the Gene for the Nonphosphorylating, NADP-Dependent Glyceraldehyde-3-Phosphate Dehydrogenase of *Streptococcus mutans*

DAVID A. BOYD, DENNIS G. CVITKOVITCH, and IAN R. HAMILTON*

Department of Oral Biology, University of Manitoba, Winnipeg, Manitoba R3E 0W3, Canada

Received 11 January 1995/Accepted 8 March 1995

We report the sequencing of a 2,019-bp region of the *Streptococcus mutans* NG5 genome which contains a 1,428-bp open reading frame (ORF) whose putative translation product had 50% identity to the amino acid sequences of the nonphosphorylating, NADP-dependent glyceraldehyde-3-phosphate dehydrogenases (GAPN) from maize and pea. This ORF is located approximately 200 bp downstream of the *ptsI* gene coding for enzyme I of the phosphoenolpyruvate:sugar phosphotransferase transport system. Mutant BCH150, in which the putative *gapN* gene had been inactivated, lacked GAPN activity that was present in the wild-type strain, thus positively identifying the ORF as the *S. mutans gapN* gene. Another strain of *S. mutans*, DC10, which contains an insertionally inactivated *ptsI* gene, still possessed GAPN activity, as did *S. salivarius* ATCC 25975, which contains an insertion element between the *ptsI* and *gapN* genes. Since the wild-type *S. mutans* NG5 lacks both glucose-6-phosphate dehydrogenase and NADH:NADP oxidoreductase activities, the NADP-dependent glyceraldehyde-3-phosphate dehydrogenase is important as a means of generating NADPH for biosynthetic reactions.

In many bacteria, the Embden-Meyerhof or glycolytic pathway functions primarily to provide energy in the form of ATP, while the hexose monophosphate pathway (HMP) operates to provide pentose for nucleic acid formation and NADPH for the biosynthesis of cellular components (18). Early studies demonstrated that some strains of *Streptococcus mutans* lack both glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) of the oxidative portion of the HMP (6). As a consequence, members of this species, long implicated as etiological agents of dental caries (2, 23), must use alternative mechanisms to generate NADPH for the use in reductive biosynthetic reactions. These bacteria overcome this metabolic dilemma by possessing two glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activities, one NAD^+ specific (EC 1.2.1.12) and the other $NADP^+$ specific (EC 1.2.1.9) (5, 31, 32). The NAD^+-specific activity would satisfy the requirements of the glycolytic pathway, while NADP-specific GAPDH is thought to be responsible for the generation of NADPH for biosynthesis. Similar enzyme activity has been shown for one strain of *Streptococcus salivarius* (SS2) but not *Streptococcus sanguis*, *Enterococcus faecalis*, *Enterococcus faecium*, or *Lactococcus lactis* (31, 32).

Early work (5) with *S. mutans* 6715 and *S. salivarius* SS2 has demonstrated that the NAD^+- and $NADP^+$-dependent GAPDH activities, unlike those of other bacteria (4, 7), are associated with distinct and separate enzymes. Partial purification of these enzymes revealed that the NAD^+-dependent enzyme carried out the conventional and reversible oxidation of glyceraldehyde-3-phosphate with the formation of 1,3-diphosphoglycerate and was totally dependent on the presence of phosphate (8). On the other hand, the $NADP^+$-dependent enzyme catalyzed the irreversible oxidation of this substrate with the formation of 3-phosphoglycerate and was not dependent on phosphate. The two enzymes were shown to have distinct molecular weights, pH optima, and inhibition responses to glyceraldehyde-3-phosphate and the reduced coenzymes, suggesting differences in regulation. This seems likely in view of the fact that *S. mutans* does not appear to contain any NADPH:NAD oxidoreductase (EC 1.6.1.1) activity (5).

We have previously cloned and sequenced the genes for HPr (*ptsH*) and enzyme I (*ptsI*), the general proteins of the phosphoenolpyruvate:sugar phosphotransferase transport system (PTS) from *S. mutans* (3). Subsequent analysis of the sequence downstream of the *ptsI* gene has revealed an open reading frame (ORF) of some 1,400 bp, and since we are interested in isolating and characterizing the genes involved in, or closely related to, the PTS in *S. mutans*, we sought to identify the activity associated with this ORF. Using the putative translation product of this ORF to search the GenBank database, we found that it had about 50% identity to the nonphosphorylating NADP-dependent GAPDHs (GAPNs) of maize and pea (19). We show here that the ORF immediately downstream of *ptsI* codes for the gene expressing GAPN activity and describe the isolation of a mutant defective in this activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth. *S. mutans* NG5 (A. Bleiweis, University of Florida, Gainesville) and BM71 (G. Bowden, University of Manitoba, Winnipeg, Manitoba, Canada) were maintained on Todd-Hewitt plates (Difco) and grown for DNA isolation, or for preparation of cell extracts, in TYE-glucose broth (1% tryptone, 0.5% yeast extract, 0.5% glucose, 17 mM K_2PO_4). *S. mutans* BCH150 and DC10 were grown as described above with the medium supplemented with 10 μg of tetracycline per ml and 10 μg of erythromycin per ml, respectively. *Escherichia coli* SURE (Stratagene), *E. coli* DH5α (GIBCO/BRL), and plasmid pBluescript SK (Stratagene) were used as previously described (3). Construction of pDB101, consisting of a 3.5-kb *Eco*RI-*Sst*I fragment of *S. mutans* NG5 genomic DNA cloned into pBluescript SK, has been described previously (3), while plasmids pDB125 and pDB123 are deletion subclones of pDB101. Plasmid pDB302 is pBluescript SK that contains a 1.7-kb *Hin*dIII-*Sau*3A fragment isolated from a recombinant λEMBL3 phage containing a 15-kb region of the *S. mutans* NG5 genome (3). Plasmid pDB123ΔPM-T was constructed by replacing a 600-bp *Pst*I-*Mun*I region from the NG5 DNA insert in pDB123 with the tetracycline resistance gene isolated as a 4.4-kb *Eco*RI-*Pst*I fragment from pDP1. Plasmid pDP1, which consists of a 4.4-kb fragment from Tn*916* and contains a gene for tetracycline resistance cloned into the *Bam*HI site of pBGS8

* Corresponding author. Phone: (204) 789-3615. Fax: (204) 888-4113.

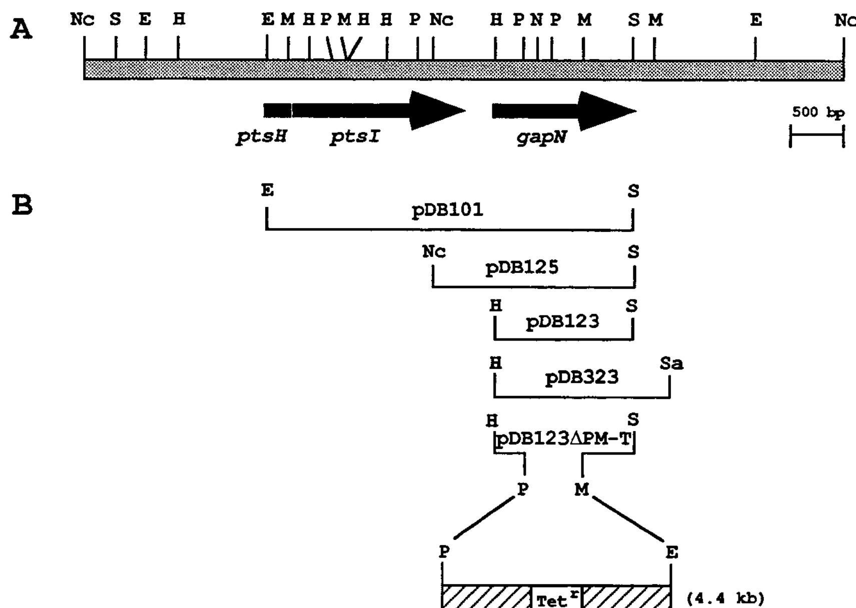

FIG. 1. (A) Restriction map of the *S. mutans* NG5 genomic *ptsH-ptsI* region showing locations of the *ptsH* and *ptsI* genes as previously determined (3) and the location of *gapN* gene (this report). (B) Restriction fragments subcloned into plasmids and used for sequencing and activity assays in *E. coli* and for constructing the *gapN* mutant *S. mutans* BCH150. Restriction sites: E, *Eco*RI; H, *Hin*dIII; M, *Mun*I; Nc, *Nco*I; N, *Nsp*V; P, *Pst*I; S, *Sst*I.

(30), was a generous gift from Robert Burne, University of Rochester, Rochester, N.Y.

Strain BCH150 was constructed by transformation of *S. mutans* BM71 with pDB123ΔPM-T that had been linearized by digestion with *Xho*I (see below). Transformants were selected on Todd-Hewitt plates containing tetracycline. Genomic DNA was isolated from two tetracycline-resistant colonies, and Southern hybridization was carried out with the insert from pDB123, the tetracycline resistance cassette from pDP1, and pBluescript. The results indicated that the insert from pDB123ΔPM-T replaced the corresponding wild-type region by double-crossover allelic exchange in both of the tetracycline-resistant colonies and that no vector sequences were present. One of the colonies was selected for further study and named *S. mutans* BCH150. *S. mutans* DC10 was constructed by a method similar to one previously described (9).

DNA methodology. *S. mutans* DNA isolation, plasmid isolation, agarose gel electrophoresis, Southern hybridizations, transformation of *E. coli*, and sequence analysis were performed as previously described (3). Transformation of streptococci was by electroporation as follows. A single colony was used to inoculate 5 ml of Todd-Hewitt broth containing 10% heat-inactivated horse serum (THBS), and the culture was incubated anaerobically at 37°C. The next day, 125 μl of the overnight culture was used to inoculate 5 ml of prewarmed THBS; this culture was grown anaerobically at 37°C until the optical density at 600 nm reached 0.25. The cells were then collected by centrifugation at 4°C, washed three times with 300 mM sucrose, and finally suspended in 100 μl of 300 mM sucrose. DNA was added to the cell suspension; after 1 min on ice, the suspension was transferred to a 0.1-cm cuvette and pulsed with a Bio-Rad Gene Pulser set at 1.25 kV and 25 μF, with a pulse controller set at 200 ohms.

Preparation of cell extracts. Cell extracts for enzyme assays were prepared as follows. *S. mutans* or *E. coli* was grown to late log phase, harvested, washed once with triethanolamine buffer (50 mM triethanolamine, 1 mM EDTA, 1 mM dithiothreitol [pH 8.0]), and the cell pellet suspended in 2 ml of triethanolamine buffer containing 0.1 μM pepstatin A and 0.1 mM phenylmethylsulfonyl fluoride (TEDPP buffer). Cells of *S. mutans* were sonicated on ice (six 30-s bursts) (Heat Systems-Ultrasonics model W375, 50% power, standard microtip) with 1 g of glass beads (0.1 to 0.15 mm) per ml, with the suspension kept on ice for 1 min between each sonication. Cells of *E. coli* were sonicated without the glass beads. Cell debris and glass beads were removed by centrifugation at 12,000 × *g* for 10 min at 4°C, and the supernatant was dialyzed overnight against 2 liters of TEDPP buffer with one change of the buffer. The dialysate was collected and centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant was stored at −70°C. Protein concentrations of the cytoplasmic preparations were determined by the method of Lowry et al. (25).

Enzyme assays. GAPN was assayed with either $NADP^+$ or NAD^+ in the reaction mixture as described by Crow and Wittenberger (8). NAD^+-dependent GAPDH (8) and G6PDH (6) were assayed as previously described. NADPH: NAD oxidoreductase (transhydrogenase) was assayed by monitoring the reduction of the NAD^+ analog acetylpyridine adenine dinucleotide at 375 nm as described by Hutton et al. (21). All assays were carried out in a total volume of 1 ml at 37°C. The DL-glyceraldehyde-3-phosphate used in the enzyme assays was prepared from DL-glyceraldehyde-3-phosphate diethyl acetal monobarium salt by conversion to the free acid followed by titration to pH 6.0 with 0.5 M triethanolamine (pH 8.3) just before use in the enzyme assays. All enzyme activities are expressed as nanomoles of NAD^+ or $NADP^+$ reduced per milligram of protein per minute.

Reagents. DL-Glyceraldehyde-3-phosphate diethyl acetal monobarium salt and acetylpyridine adenine dinucleotide were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals and enzymes were obtained either from GIBCO/BRL, Sigma, or Fisher Scientific Limited.

Computer-aided analysis. The BLAST program (1) was used to search the GenBank database. Similarities were assessed by using the ALIGN program (11), multiple sequence alignments were carried out with the CLUSTALV program (20), and the Δ*G* values and structures of transcriptional terminator regions were analyzed by the MULFOLD program (22).

Nucleotide sequence accession number. The sequence shown in Fig. 2 is available in the GenBank database under accession number L38521.

RESULTS

Nucleotide sequence of the *gapN* gene. We have previously cloned a 3.5-kb *Eco*RI-*Sst*I fragment from the *S. mutans* NG5 genome (pDB101) that contains the ORFs for the *ptsH* and *ptsI* genes (3) (Fig. 1). The completed sequence analysis of pDB101 revealed an ORF in the same orientation as the *ptsI* gene starting with a TTG codon at position 233 and extending 1,428 bp to a stop codon TAA beginning at position 1658 (Fig. 2). There is another in-frame TTG codon at position 323 before the first in-frame ATG codon is encountered at position 341 (Fig. 2).

A search of the GenBank database with the putative translation product of the 1,428-bp ORF revealed that it had about 50% identity to GAPNs (encoded by the *gapN* gene) of maize and pea identified by Habenicht and coworkers (19) (Fig. 3). As an alignment of the *S. mutans* NG5 putative GAPN with those from pea and maize (Fig. 3) revealed several shared residues near the N terminus, it is likely that the TTG codon at position 233 is the true start codon for the *S. mutans* gene. Further indication of this is that the TTG codon (position 233) is preceded 8 bp away from a putative ribosome binding site, AGGAG at positions 220 to 224, while downstream, the TTG

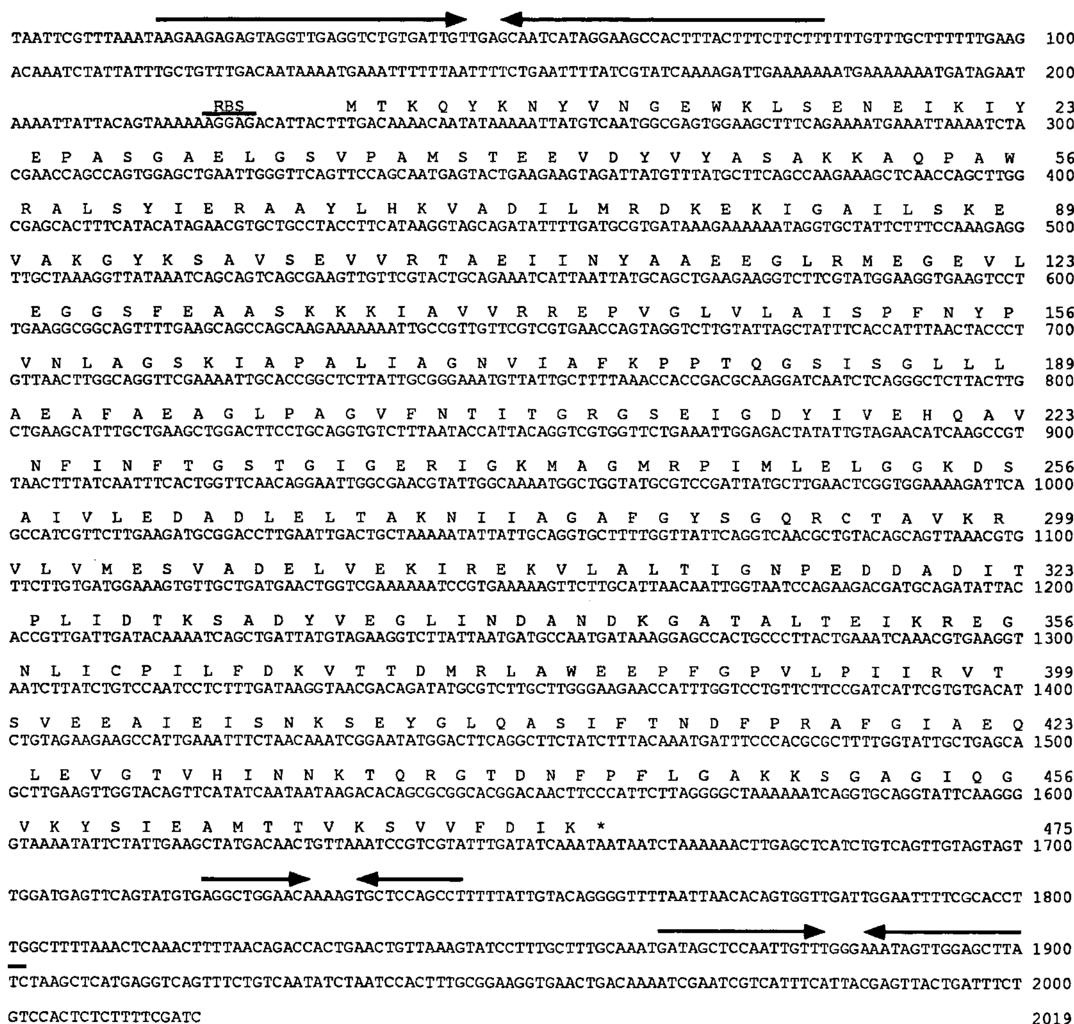

FIG. 2. Nucleotide sequence of the *S. mutans* NG5 *gapN* gene and its deduced amino acid sequence. Putative transcriptional terminator-like regions are indicated by inverted arrows. The sequence begins with the first nucleotide of the *ptsI* stop codon, with the sequence from positions 1 to 140 previously reported as part of GenBank accession number L15191 (3). Gaps are not counted in the numbering scheme. RBS, ribosome binding site.

codon (position 323) and ATG codon (position 341) are not preceded by sequences resembling ribosome binding sites (Fig. 2). The 1,428-bp putative *gapN* ORF has a putative translation product of 475 amino acids with a molecular mass of 51,145 Da. Base composition analysis of the 1,428-bp *gapN* ORF showed it to be 59.8% A+T, which is similar to the values for *S. mutans* NG5 *ptsI* and *ptsH* genes, which are 58.4 and 60.6%, respectively (3).

We sequenced 359 bp downstream from the stop codon of the putative *gapN* gene and could not detect any other ORFs of significance. This region does, however, contain at least two regions of dyad symmetry which could play a role in transcriptional termination. The first one, located at bases 1720 to 1745 (Fig. 2), has a ΔG of -12.1 kcal mol^{-1} (-50.7 kJ mol^{-1}) and is followed by a short run of T's characteristic of rho-independent terminators (10). The second region may play a role in transcriptional regulation of a downstream gene that is in the opposite orientation to the putative *gapN* gene.

Insertional inactivation of GAPN. To determine if the putative *gapN* gene was functional, we mutated the gene in vitro by replacing a small internal fragment of it with a tetracycline resistance gene (pDB123ΔPM-T; Fig. 1) and then used this mutated gene to transform *S. mutans* BM71 to tetracycline resistance. The mutated gene replaced the wild-type gene in a tetracycline-resistant transformant (BCH150) by double-crossover allelic exchange as determined by Southern analysis (data not shown). We used BM71 to construct the *gapN* mutant, since this strain could be made competent for DNA uptake whereas NG5 could not. We had previously carried out Southern hybridizations on various restriction digests of BM71 DNA with the cloned NG5 DNA from the *ptsI-gapN* region and had shown that the genetic organization in this region of the two strains was identical (data not shown).

Growth of the mutant BCH150 was slower than growth of the wild-type strain BM71 on both solid and liquid media. For example, in TYE-glucose broth, anaerobic cultures of BCH150 were characterized by long lag periods, with an average doubling time of 200 ± 20 min (data not shown). This doubling time was threefold longer than that for BM71 (60 ± 12 min). In addition, while the final terminal pH values of the cultures were similar, i.e., 4.1 ± 0.05 for BM71 and 4.2 ± 0.05 for BCH150, the final cell yield of stationary-phase BCH150 cultures was only 60% of that of the BM71 (data not shown). BCH150 was also unable to grow in the presence of oxygen.

```
Ssal                 MAKQYKNYVNGEWKTSENE--ITIYAPANGEELGSVPAMSQAEVDEVYAAAKAALPAWRALSYAERAAYLHKAAD  73
NG5                  MTKQYKNYVNGEWKLSENE--IKIYEPASGAELGSVPAMSTEEVDYVYASAKKAQPAWRALSYIERAAYLHKVAD  73
Psat      MAATGVLAEIIDGDVYKYYADGEWNKSTSGKSVAIINPTTRKPQYKVQACSQEEVNKVMDSAKSPQKSWAKTPLWKRAELLHKAAA  86
Zmays   MALAGTGVFAEILDGEVYRYYADGEWRTSASGKSVAIVNPTTRKTQYRVQACTQEEVNKAMDAAKVAQKAWARTPLWKRADVLHKAAA  88
                         *. * .***  * .   . * .*..      * * .  **.    .** .  .*   .   **  *** *

NG5     ILMRDKEKIGAILSKEVAKGYKSAVSEVVRTAEIINYAAEEGLRM--EGEVLEGGSFEAASKKKIAVVRREPVGLVLAISPFNYPVNL 159
Psat    ILKEHKAAIAECLVKEIAKPAKDAVTEVVRSGDLVSYCAEEGVRILGEGKFLVSDSFPGNERTKYCLTSKIPLGVILAIPPFNYPVNL 174
Zmays   ILKEHKAPIAECLVKEIAKPAKDAVSEVVRSGDLVSYTAEEGVRILGEGKLVVSDSFPGNERNKYCLSSKIPLGVVLAIPPFNYPANL 176
        **   .*  *.  * **.**  * **.****......* ****.*.  **   . ..**  .  . *   .  . *.*..***.***** **

NG5     AGSKIAPALIAGNVIAFKPPTQGSISGLLLAEAFAEAGLPAGVFNTITGRGSEIGDYIVEHQAVNFINFTGSTGIGERIGKMAGMRPI 247
Psat    AVSKIAPALIAGNSIVLKPPTQGAVAALHMVHCFHLAGFPKGLISCVTGKGSEIGDFLTMHPGVNCISFTGG-DTGIAISKKSGMIPL 261
Zmays   AGSKIGPALIAGNALVLKPPTQGAVAALHMVHCFHLAGFPKGLISCVTGKGSEIGDFLTMHPGVNCISFTGG-DTGIAISKKAGMVPL 263
        * ***.*******  . .******....*  .  . *   **.* *... .**.******..   * .** *.***. . *  *.* .** *.

NG5     MLELGGKDSAIVLEDADLELTAKNIIAGAFGYSGQRCTAVKRVLVMESVADELVEKIREKVLALTIGNPEDDADITPLIDTKSADYVE 335
Psat    QMELGGKDACIVLEDADLDLVAANIIKGGFSYSGQRCTAVKVVLVMESVADALVEKVKVKVAKLSVGPPEDDSDITPVVSESSANFIE 349
Zmays   QMELGGKDACIVLEDADLDLVSANIVKGGFSYSGQRCTAVKVVLIMESIADAVVQKVNAKLAKLKVGPPEDDSDITPVVTESSANFIE 351
         .******. ********.* . **. *.*.********** **.***.** .*.*.  *.  * .* ****.****..   **...*

NG5     GLINDANDKGATALTEIKREGNLICPILFDKVTTDMRLAWEEPFGPVLPIIRVTSVEEAIEISNKSEYGLQASIFTNDFPRAFGIAEQ 423
Psat    GLVNDAKEKGATFCQEYKREGNLIWPLLLDNVRPDMRIAWEEPFGPVLPVIRINSVEEGIHHCNASNFGLQGCVFTKDINKAIMISDA 437
Zmays   GLVMDAKEKGATFCQEYRREGNLIWPLLLDHVRPDMRIAWEEPFGPVLPVIRINSVEEGIHHCNASNFGLQGCIFTRDINKAILISDA 439
        **. **..****    * .****** *.*.* *   ***.***********.**. ****.*.  * *..***. .** *. .*. *..

NG5     LEVGTVHINNKTQRGTDNFPFLGAKKSGAGIQGVKYSIEAMTTVKSVVFDIK        475
Psat    MESGTVQINSAPARGPDHFSFQGIKDSGIGSQGITNSINMMTKVKTTVINLPSPSYTMG 496
Zmays   METGTVQINSAPARGPDHFSFQGLKDSGIGSQGITNSINMMTKVKSTVINLPSPSYTMG 498
        .* ***.**.    ** *.*.* * * ** * **.  **. ** **. *
```

FIG. 3. Alignment of the amino acid sequences of *S. mutans* NG5 GAPN (NG5) with the GAPNs of *S. salivarius* 25975 (Ssa1), pea (*Pisum sativum* [Psat]), and maize (*Zea mays* [Zmays]). The *gapN* gene of *S. salivarius* has been only partially sequenced (24). Under the sequences, asterisks indicate identical residues and dots indicate similar residues. Residue numbers are indicated; gaps are not counted in the numbering scheme.

Enzyme activity. Assays for GAPN activity were carried out with $NADP^+$, using cell extracts from *S. mutans* NG5, BM71, and BCH150. The results showed that NG5 and BM71 had similar levels of GAPN activity, while BCH150 had less than 2% of the wild-type activity of the enzyme (Table 1). The activity of NAD^+-dependent GAPN in wild-type strains was 1 to 3% of $NADP^+$-dependent activity. Consequently, we designated the ORF in *S. mutans* NG5 as the *gapN* gene encoding a nonphosphorylating, $NADP^+$-dependent GAPDH. Assays for the phosphorylating, NAD^+-dependent GAPDH showed that NG5 and BM71 had similar levels, while BCH150 had approximately 50% more activity than the wild-type strains (Table 1).

Earlier work has demonstrated that strains of *S. mutans* and *S. salivarius* that possess GAPN lacked both the oxidative portion of the HMP and NADPH:NAD^+ oxidoreductase (transhydrogenase) (5, 6). To determine if the strains used in our study possessed this part of the HMP, we assayed them for the first oxidative enzyme of the pathway, G6PDH. G6PDH activity in the test strains was very low (Table 1), in keeping with earlier results (6), and 22-fold lower than that in *S. sanguis* (5). To determine whether NADPH could be generated by a transhydrogenase-catalyzed reaction, BM71 and BCH150 were assayed for this activity. No activity could be detected (data not shown).

To determine if GAPN activity is dependent on the expression of the *ptsI* gene, we assayed for GAPN activity in *S. mutans* DC10, a mutant in which the *ptsI* gene of BM71 has been inactivated by the insertion of an erythromycin resistance gene (9). The assay revealed that the *gapN* gene is expressed, although the levels are about 60 to 70% of those of BM71 (Table 1). This finding indicated that the *gapN* gene can be expressed independently of the presence of a functional enzyme I. DC10 showed levels of GAPDH activity similar to those of BM71 and NG5 (Table 1).

TABLE 1. GAPN, GAPDH, and G6PDH activities in cell extracts of *S. salivarius* and wild-type and mutant strains of *S. mutans*

Organism	Mean enzyme activity (nmol/min/mg of protein ± SE)			
	GAPN		GAPDH, NAD^+	G6PDH, $NADP^+$
	$NADP^+$	NAD^+		
Streptococci				
S. mutans NG5	164 ± 26	4.3 ± 0.7	35 ± 4	2.2 ± 0.3
S. mutans BM71	179 ± 47	7.4 ± 0.7	43 ± 5	2.6 ± 1.0
S. mutans BCH150	2.5 ± 0.4	5.5 ± 0.4	77 ± 6	0.8 ± 0.3
S. mutans DC10	97 ± 24	1.5 ± 0.1	59 ± 11	0.6 ± 0.3
S. salivarius 25975	498 ± 67	5.5 ± 1.5	88 ± 9	3.5 ± 0.6
E. coli SURE with plasmid:				
pDB101	7,800 ± 760	2.6 ± 0.4	—[a]	—
pDB125	5,400 ± 702	2.4 ± 0.6	—	—
pDB123	0.5 ± 0.2	2.5 ± 0.6	—	—
pBluescript	1.5 ± 0.4	4.2 ± 3.4	—	—

[a] —, not done.

Previously, *S. salivarius* SS2 was shown to possess GAPN activity (5); recently the *ptsH-ptsI* region of *S. salivarius* 25975 was sequenced (15, 17), and an insertion element, IS*1139*, has been identified downstream of the *ptsI* gene followed by an ORF coding for a putative dehydrogenase (24). By comparison of the putative translation product of the sequenced region of this ORF with the GAPN of *S. mutans* NG5, we showed that this ORF is the *gapN* gene of *S. salivarius* 25975, as the two proteins have 84% identity in this region (Fig. 3). It has also been shown in *S. salivarius* 25975 that no transcripts originating from *ptsH-ptsI* extend past IS*1139* (16). Assays for GAPN activity in *S. salivarius* 25975 revealed levels of activity 40% higher than those of BM71 and NG5 (Table 1), suggesting that the *gapN* gene can be expressed from its own promoter. *S. salivarius* 25975 exhibited levels of GAPDH activity 50% higher than those of BM71 and NG5 (Table 1). These higher levels of GAPN and GAPDH activities in *S. salivarius* 25975, which has a functional PTS, are mirrored in its growth rate, which is 1.5 to 2 times faster than those of BM71 and NG5 (data not shown).

Expression of GAPN in *E. coli*. To determine if the *gapN* gene could be expressed in *E. coli*, which has no GAPN activity, we transformed *E. coli* SURE with pDB101 and pDB125 and assayed cell extracts for activity. Both plasmids contain the complete *gapN* gene, and pDB101 also contains the complete *ptsI* gene, which we have shown previously is expressed from this construct in *E. coli* (3). The assays revealed high levels of GAPN activity in cells harboring pDB101 or pDB125 compared with the negative control, pBluescript, which showed negligible activity (Table 1). The extremely high levels of activity in these *E. coli* strains is likely due in part to the dosage effect of the cloned gene in the high-copy-number pBluescript vector, as well as more efficient recovery of protein from gram-negative *E. coli* with the extraction procedure used. We were also interested in whether *E. coli* SURE harboring pDB123 had any GAPN activity, as the insert in pDB123 begins at a *Hin*dIII site (bases 272 to 277; Fig. 2) 68 bp upstream of the first ATG codon of the *gapN* ORF (bases 341 to 343; Fig. 2) and continues to the end of the gene as for pDB101 and pDB125 (Fig. 1). This construct conferred no GAPN activity to the extract of the *E. coli* harboring the plasmid (Table 1), showing that the *Hin*dIII site either lies within the *gapN* coding sequence or lies downstream of the *gapN* promoter, or both. The ability of the *S. mutans gapN* gene to be expressed in *E. coli* could aid in purification of the protein, which could be used in determination of the N-terminal sequence.

DISCUSSION

The data reported here show that the putative translation product of an ORF located 233 bp downstream of the *ptsI* gene has 50% identity to the GAPNs of maize and pea reported by Habenicht et al. (19) (Fig. 3). This protein is not related to the classical NAD-specific GAPDH of glycolysis but is a member of the aldehyde dehydrogenase superfamily, which contains specialized and nonspecialized aldehyde dehydrogenases of both prokaryotes and eukaryotes that share between 20 and 40% identity (19, 28). Among eukaryotes, GAPN has been found only among photosynthetic organisms, including algae, in which it is found in the cytosol, catalyzing the irreversible, phosphate-independent oxidation of chloroplast-produced glyceraldehyde-3-phosphate to 3-phosphoglycerate, thereby generating NADPH for biosynthetic processes. In prokaryotes, studies have shown that certain strains of streptococci possess an $NADP^+$-dependent GAPDH (5), and subsequent purification and kinetic studies revealed the enzyme to catalyze a reaction identical to that catalyzed by the GAPN of photosynthetic eukaryotes (8). To show whether the ORF in *S. mutans* was the functional *gapN* gene, we used an internal region of the cloned ORF to construct a mutant, BCH150, in which the gene was insertionally inactivated (Fig. 1). Assays for GAPN activity clearly showed that BCH150 had negligible activity compared with both BM71 and NG5 (Table 1). Thus, we designated this ORF as the *gapN* gene of *S. mutans* coding for the GAPN protein. To our knowledge, this is the first characterization of this gene in prokaryotes and only the third complete nucleotide sequence to be reported, the other two being the cDNA sequences of the *gapN* genes of maize and pea (19). There has been a report of an $NADP^+$-dependent GAPDH activity in *Pseudomonas aeruginosa* that has been separated from an NAD-specific activity; however, it has not been genetically characterized (29).

Interestingly, on the basis of the sequence analysis of *gapN* (Fig. 2) and alignment of the translation product with the plant GAPNs (Fig. 3), we postulated that the start codon is not the normal ATG but rather a rare TTG. TTG has previously been shown to be used as an initiation codon in gram-positive bacteria, e.g., for the β-lactamase genes of *S. aureus* (26) and *Bacillus cereus* (27), and for the L-(−)-lactate dehydrogenase gene of *Lactobacillus plantarum* (13). Assuming that the gene begins with the TTG codon, the subunit size of the *S. mutans* GAPN would be 51 kDa, which is similar to the size of eukaryotic GAPN subunits (19). However, where the eukaryotic GAPNs have been reported to be homotetramers with the native enzyme thus being about 200 kDa, the *S. mutans* enzyme has been reported to have a size of 350 kDa in its native state (8). If the *S. mutans* native enzyme structure consists of identical subunits, it would have to contain at least six of these subunits. N-terminal sequencing of purified *S. mutans* GAPN will be needed to determine if the TGG codon described above is the true start codon.

The spacing between the *ptsI* and *gapN* genes (230 bp) and the fact that GAPN function is unrelated to the PTS indicate that the *gapN* gene is not part of the *ptsH-ptsI* transcriptional unit, a fact confirmed by the presence of GAPN activity in *S. salivarius* 25975. Transcript analysis of strains of *S. salivarius* without IS*1139* in this region will be needed to determine if *gapN* can be transcribed with *ptsI* in those strains. *S. mutans* DC10 also possesses GAPN activity (Table 1) despite a 1.8-kb insert in the *ptsI* gene (9). In *S. mutans* NG5, we have identified a putative *ptsI* terminator (Fig. 2) with a ΔG of −22.6 kcal mol^{-1} (−94.7 kJ mol^{-1}), a more stable structure than the *ptsI* terminator in IS*1139* of *S. salivarius*, which terminates all transcripts reaching it (16). These data taken together indicate that like the *S. salivarius* 25975 GAPN, the *S. mutans* GAPN can be transcribed from its own promoter and is not part of the *ptsH-ptsI* transcriptional unit. As we have detected no obvious ORFs within 359 bp downstream of *gapN*, the gene appears to be monocistronic; however, transcriptional analysis of this region in *S. mutans* will be needed to determine if *gapN* can be transcribed with *ptsI* under some conditions.

Brown and Wittenberger (5, 6) have previously shown that several strains of *S. mutans* and a strain of *S. salivarius* which possess GAPN lack the two oxidative enzymes of the HMP, G6PDH and 6PGDH. In addition, they showed that several related bacteria, *E. faecalis*, *E. faecium*, *S. sanguis*, and *L. lactis*, which possess G6PDH and 6PGDH lack GAPN activity. We have been unable to detect NADH:NADP oxidoreductase activity (transhydrogenase) in *S. mutans*, confirming earlier work by Brown and Wittenberger (5). It is apparent that the physiological role of GAPN is to provide NADPH for biosynthetic reactions, which in other bacteria would be provided by the

HMP. We have shown that GAPN is not essential for cell survival by isolating a mutant strain, *S. mutans* BCH150, in which the *gapN* gene has been inactivated and in which no G6PDH could be detected. The isolation of BCH150 indicates that *S. mutans* possesses alternate means of producing NADPH or that the cell has no absolute requirement for the reduced coenzyme. In *S. salivarius* 25975, genes for the first three enzymes of the tricarboxylic acid cycle, citrate synthase, aconitase, and isocitrate dehydrogenase, have been identified upstream of the *ptsH* gene (12, 14). Isocitrate dehydrogenase, encoded by a functional *icd* gene 200 bp upstream of the *ptsH* gene (14), requires $NADP^+$, and thus NADPH is produced during the oxidation of isocitrate to 2-ketoglutarate. It is likely that the genetic organization upstream of the *ptsH* in *S. mutans* is the same as that in *S. salivarius* so that a functional *icd* gene would provide a source of NADPH in the GAPN mutant, BCH150. If this alternate means of generating NADPH is functional in *S. mutans*, it would appear to be less efficient than GAPN, as BCH150 exhibited a slower growth rate and achieved lower cell yields compared with similarly grown BM71.

ACKNOWLEDGMENT

This research was supported by operating grant MT-3546 to I.R.H. from the Medical Research Council of Canada.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Bowden, G. H. W.** 1991. Which bacteria are cariogenic in humans?, p. 266–286 *In* N. W. Johnson (ed.), Dental caries, vol. 1. Markers of high and low risk groups and individuals. Cambridge University Press, Cambridge.
3. **Boyd, D. A., D. G. Cvitkovitch, and I. R. Hamilton.** 1994. Sequence and expression of the HPr (*ptsH*) and enzyme I (*ptsI*) genes of the phosphoenolpyruvate-dependent phosphotransferase transport system from *Streptococcus mutans*. Infect. Immun. **62:**1156–1165.
4. **Brenneman, F. N., and W. A. Volk.** 1959. Glyceraldehyde phosphate activity with triphosphopyridine nucleotide and with diphosphopyridine nucleotide. J. Biol. Chem. **234:**2443–3447.
5. **Brown, A. T., and C. L. Wittenberger.** 1971. The occurrence of multiple glyceraldehyde-3-phosphate dehydrogenases in cariogenic bacteria. Biochem. Biophys. Res. Commun. **43:**217–224.
6. **Brown, A. T., and C. L. Wittenberger.** 1971. Mechanism for regulating the distribution of glucose carbon between the Embden-Meyerhof and hexose-monophosphate pathways in *Streptococcus faecalis*. J. Bacteriol. **106:**456–467.
7. **Burchall, J. J., R. A. Niederman, and M. J. Wolin.** 1964. Amino group formation and glutamate synthesis in *Streptococcus bovis*. J. Bacteriol. **88:** 1038–1044.
8. **Crow, V. L., and C. L. Wittenberger.** 1979. Separation and properties of NAD^+-and $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenases from *Streptococcus mutans*. J. Biol. Chem. **254:**1134–1142.
9. **Cvitkovitch, D. G., D. A. Boyd, and I. R. Hamilton.** 1995. Glucose transport by a mutant of *Streptococcus mutans* unable to transport sugars via the phophoenolpyruvate:sugar phosphotransferase system. J. Bacteriol. **177:**2251–2258.
10. **d'Aubenton, C. Y., E. Brody, and C. Thermes.** 1990. Prediction of rho-independent *Escherichia coli* terminators. J. Mol. Biol. **216:**835–858.
11. **Dayhoff, M. O., W. C., Barker, and L. T. Hunt.** 1983. Establishing homologies in protein sequences. Methods Enzymol. **91:**524–545.
12. **Desgagnes, R., G. Gagnon, and M. Frenette.** 1993. Cloning, sequencing and characterization of the *icd* gene that codes for the *Streptococcus salivarius* isocitrate dehydrogenase, abstr. K-40. *In* Abstracts of the 93rd General Meeting of the American Society for Microbiology 1993. American Society for Microbiology, Washington, D.C.
13. **Ferain, T., D. Garmyn, N. Bernard, P. Hols, and J. Delcour.** 1994. *Lactobacillus plantarum ldhL* gene: overexpression and deletion. J. Bacteriol. **176:** 596–601.
14. **Frenette, M. (Université Laval).** 1995. Personal communication.
15. **Gagnon, G., C. Vadeboncoeur, and M. Frenette.** 1993. Phosphotransferase system of *Streptococcus salivarius*: characterization of the *ptsH* gene and its product. Gene **136:**27–34.
16. **Gagnon, G., C. Vadeboncoeur, and M. Frenette.** 1994. Transcriptional regulation of the *pts* operon in *Streptococcus salivarius* ATCC 25975, abstr. T35. *In* Program and Abstracts of the 4th International Conference on Streptococcal Genetics. American Society for Microbiology, Washington, D.C.
17. **Gagnon, G., C. Vadeboncoeur, R. C. Levesque, and M. Frenette.** 1992. Cloning, sequencing and expression in *Escherichia coli* of the *ptsI* gene encoding enzyme I of the phosphoenolpyruvate phosphotransferase transport system from *Streptococcus salivarius*. Gene **121:**71–78.
18. **Gottschalk, G.** 1986. Bacterial metabolism. Springer-Verlag, New York.
19. **Habenicht, A., U. Hellman, and R. Cerff.** 1994. Non-phosphorylating GAPDH of higher plants is a member of the aldehyde dehydrogenase superfamily with no sequence homology to phosphorylating GAPDH. J. Mol. Biol. **237:**165–171.
20. **Higgins, D. G., A. J. Bleasby, and R. Fuchs.** 1991. CLUSTALV: improved software for multiple sequence alignment. Comp. Appl. Biosci. **8:**189–191.
21. **Hutton, M., J. M. Day, T. Bizouarn, and J. B. Jackson.** 1994. Kinetic resolution of the reaction catalysed by proton-translocating transhydrogenase from *Escherichia coli* as revealed by experiments with analogues of the nucleotide substrates. Eur. J. Biochem. **219:**1041–1051.
22. **Jaeger, J. A., D. H. Turner, and M. Zuker.** 1989. Predicting optimal and suboptimal secondary structure for RNA. Methods Enzymol. **183:**281–306.
23. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. **50:**353–380.
24. **Lortie, L.-A., G. Gagnon, and M. Frenette.** 1994. IS1139 from *Streptococcus salivarius*: identification and characterization of a insertion sequence-like element related to mobile DNA elements from Gram-negative bacteria. Plasmid **32:**1–9.
25. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
26. **McLaughlin, J. R., C. L. Murray, and J. C. Rabinowitz.** 1981. Unique features in the ribosome binding site sequence of the Gram-positive *Staphylococcus aureus* β-lactamase gene. J. Biol. Chem. **256:**11273–11282.
27. **Mézes, P. S. F., R. W. Blacher, and J. O. Lampen.** 1985. Processing of *Bacillus cereus* 569/H β-lactamase I in *Escherichia coli* and *Bacillus subtilis*. J. Biol. Chem. **260:**1218–1223.
28. **Michels, S., S. Scagliarini, F., Della Seta, C. Carles, M. Riva, P., Trost, and G. Bralant.** 1994. Arguments against a close relationship between non-phosphorylating and phosphorylating glyceraldehyde-3-phosphate dehydrogenases. FEBS Lett. **339:**97–100.
29. **Rivers, D. B., and W. T. Blevins.** 1987. Multiple enzyme forms of glyceraldehyde dehydrogenase in *Pseudomonas aeruginosa* PAO. J. Gen. Microbiol. **133:**3159–3164.
30. **Spratt, B. G., P. J. Hedge, S. te Heesen, A. Hedelman, and J. K. Broome-Smith.** 1986. Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8, and pEMBL9. Gene **41:**337–342.
31. **Wittenberger, C. L., and V. L. Crow.** 1976. Regulation of carbon flow within and between metabolic pathways in certain species of *Streptococcus*, p. 617–629. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Microbial aspects of dental caries. Special Suppl. Microbiol. Abstr. vol. III. Information Retrieval Inc., Washington, D.C.
32. **Yamada, T., and J. Carlsson.** 1973. Phosphoenolpyruvate carboxylase and ammonium metabolism by oral streptococci. Arch. Oral Biol. **18:**799–812.