JOURNAL OF BACTERIOLOGY, Feb. 1997, p. 650–655 Vol. 179, No. 3
0021-9193/97/$04.00+0

Role of the Citrate Pathway in Glutamate Biosynthesis by *Streptococcus mutans*

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Received 29 July 1996/Accepted 9 November 1996

In work previously reported (J. A. Gutierrez, P. J. Crowley, D. P. Brown, J. D. Hillman, P. Youngman, and A. S. Bleiweis, J. Bacteriol. 178:4166–4175, 1996), a Tn*917* transposon-generated mutant of *Streptococcus mutans* JH1005 unable to synthesize glutamate anaerobically was isolated and the insertion point of the transposon was determined to be in the *icd* gene encoding isocitrate dehydrogenase (ICDH). The intact *icd* gene of *S. mutans* has now been isolated from an *S. mutans* genomic plasmid library by complementation of an *icd* mutation in *Escherichia coli* host strain EB106. Genetic analysis of the complementing plasmid pJG400 revealed an open reading frame (ORF) of 1,182 nucleotides which encoded an enzyme of 393 amino acids with a predicted molecular mass of 43 kDa. The nucleotide sequence contained regions of high (60 to 72%) homology with *icd* genes from three other bacterial species. Immediately 5′ of the *icd* gene, we discovered an ORF of 1,119 nucleotides in length, designated *citZ*, encoding a homolog of known citrate synthase genes from other bacteria. This ORF encoded a predicted protein of 372 amino acids with a molecular mass of 43 kDa. Furthermore, plasmid pJG400 was also able to complement a citrate synthase (*gltA*) mutation of *E. coli* W620. The enzyme activities of both ICDH, found to be NAD^+ dependent, and citrate synthase were measured in cell extracts of wild-type *S. mutans* and *E. coli* mutants harboring plasmid pJG400. The region 5′ from the *citZ* gene also revealed a partial ORF encoding 264 carboxy-terminal amino acids of a putative aconitase gene. The genetic and biochemical evidence indicates that *S. mutans* possesses the enzymes required to convert acetyl coenzyme A and oxalacetate to α-ketoglutarate, which is necessary for the synthesis of glutamic acid. Indeed, *S. mutans* JH1005 was shown to assimilate ammonia as a sole source of nitrogen in minimal medium devoid of organic nitrogen sources.

Streptococcus mutans is the principal etiological agent of dental caries (18, 41). This organism is a normal inhabitant of dental plaque; however, under the appropriate environmental conditions, it is able to generate acidic end products which cause the dissolution of tooth enamel (18, 42). The dietary intake of sugars, especially sucrose, has been associated unequivocally with the virulence of *S. mutans*; however, under most situations, sugars do not appear to be the limiting nutrients in saliva (6). Although the precise concentrations of individual nutrients in plaque are not known, it has been established that *S. mutans* has simple nutritional requirements (5, 38, 39) and, in a mixed plaque community, is able to grow with the components of saliva as its sole sources of carbon and nitrogen (9).

Results of early work suggesting that *S. mutans* has simple nutritional requirements demonstrated that some strains were able to grow in a minimal medium supplemented with only a few amino acids (5, 39). It was later demonstrated that, when grown anaerobically, some strains of *S. mutans* can be cultured in vitro on simple growth media containing no amino acids with ammonia as the sole source of nitrogen (38).

It was demonstrated that this organism uses glutamate dehydrogenase (EC 1.4.1.4) to assimilate the ammonia required for the subsequent biosynthesis of all amino acids (16). It was also shown, by using mutants defective in expression of this enzyme, that *S. mutans* is able to fix free ammonia by the activities of glutamine synthetase (EC 6.3.1.2) and glutamate synthase (EC 2.6.1.53) (38). At the time of this early work, the mechanism utilized by *S. mutans* to synthesize α-ketoglutarate, the substrate of glutamate dehydrogenase, was not known (38).

Among aerobic bacteria able to grow on acetate as the sole carbon source, isocitrate dehydrogenase (ICDH) has an important regulatory function. ICDH controls the flow of isocitrate between the Krebs tricarboxylic acid (TCA) cycle and the glyoxylate bypass (23, 24). In addition to its catabolic function, ICDH also plays a key role in the citrate pathway of glutamate biosynthesis by converting isocitrate to α-ketoglutarate for subsequent conversion to glutamate by glutamate dehydrogenase (24, 31). The existence of ICDH in *Streptococcus salivarius* has been documented previously (10), but its role in glutamate biosynthesis in oral streptococci has not been demonstrated.

It has been established that *S. mutans* is able to synthesize the primary substrates of the citrate pathway by the production of oxalacetate from phosphoenolpyruvate (PEP) and CO_2 via PEP carboxylase (EC 4.1.1.31) (43). It is also known that *S. mutans* can generate acetyl coenzyme A (acetyl-CoA) by the activity of pyruvate dehydrogenase (EC 1.2.1.51) under aerobic conditions (7) and by the activity of pyruvate formate-lyase (EC 2.3.1.54) anaerobically (1). Since glutamate biosynthesis in *S. mutans* occurs only under anaerobic conditions (5, 38, 39), the source of acetyl-CoA for use in this pathway is likely pyruvate formate-lyase. An outline of the proposed use of the citrate pathway under anaerobic conditions and the reactions utilized by *S. mutans* is illustrated in Fig. 1.

To gain insight into the mechanisms and regulation of ammonia assimilation and glutamate biosynthesis in *S. mutans*, we utilized a transposon mutagenesis-marker rescue system to isolate an auxotrophic mutant which required glutamate as a nutritional supplement (17). This technique allowed recovery of an inactivated gene fragment, which, by nucleotide sequence

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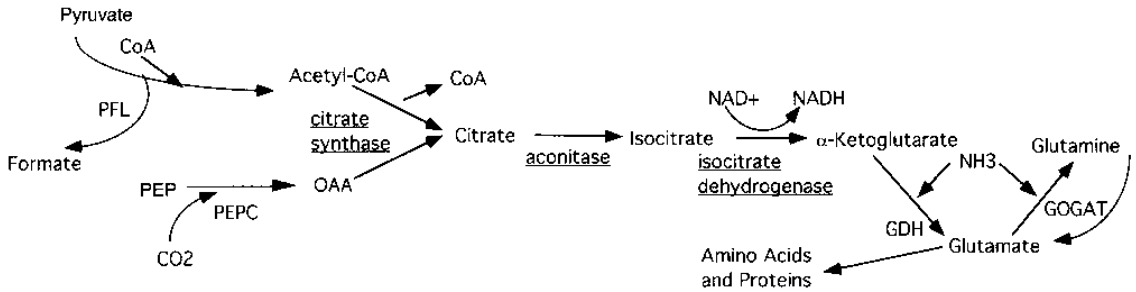

FIG. 1. Proposed use of the citrate pathway by *S. mutans* for the anaerobic synthesis of glutamate and the assimilation of ammonia. The enzymes introduced in this paper are underlined. The other enzyme activities have been described previously. Abbreviations: PFL, pyruvate formate-lyase; PEPC, PEP carboxylase; OAA, oxalacetate; GDH, glutamate dehydrogenase; GOGAT, glutamine synthetase-glutamate synthase pathway.

analysis and comparison with other known sequences, exhibited a high degree of homology with ICDH-encoding genes (*icd*) of other organisms.

This paper describes the cloning, nucleotide sequence, and expression of the intact *S. mutans icd* gene in *Escherichia coli* and demonstrates linkage on the *S. mutans* chromosome with genes encoding citrate synthase (*citZ*) and aconitase (*acn*). We also demonstrate ICDH and citrate synthase activity in *S. mutans* and describe genetic and biochemical evidence for the use of the citrate pathway in the biosynthesis of glutamate by *S. mutans.*

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5α [ϕ80d *lacZΔM15 endA1 recA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1* λ$^-$ *gyrA relA1* F$^-$ Δ(*lacZYA-argF*) U169], used as a host strain for the maintenance of plasmids in this study, was grown in Luria-Bertani (LB) broth (26) and on LB plates containing ampicillin (100 μg ml^{-1}) when required. *E. coli* MC1061 [Δ(*araA-leu*)*7697 araΔ139* Δ(*codB-lac*)3 *galK16 galE15* λ$^-$ *mcrA0 relA1 rpsl150*(*strA*) *spoT1 mcrB9999 hsdR2*] was used as a host for plasmids containing the erythromycin resistance gene from Tn*917*. *E. coli* EB106 [λ$^-$ *icd-11 dadA1*(op) *trpA62 trpE61 tnaA5*] (2) was used as a host for cloning the intact *S. mutans icd* gene and was grown in M63 medium (3) supplemented with 10 mM glucose, 0.02 mM thiamine, 1 mM L-tryptophan, and, when necessary, 3 mM L-glutamate. *E. coli* W620 (*thi-1 pyrD36 gltA6 galK30 rpsL129*) (34) was used as a host to demonstrate *citZ* complementation of the *gltA* mutation and was grown on M63 medium supplemented with 0.02 mM thiamine and, when necessary, 3 mM L-glutamate. The naturally transformable *S. mutans* strain NG-8 has been described previously (17). *S. mutans* JH1005 was kindly provided by J. D. Hillman, University of Florida, and was utilized as the parent strain in the isolation of the *icd* mutant AX1. The glutamate-requiring auxotroph AX1 was isolated by Tn*917* transposon mutagenesis utilizing the replication-conditional plasmid pTV1-OK as described previously (17). *S. mutans* strains were maintained and grown in Todd-Hewitt broth (Difco) supplemented with 0.3% yeast extract (THBYE) or on THBYE–1.5% agar plates. A streptococcal minimal medium devoid of amino acids (SMM) described by St. Martin and Wittenberger (38) was also utilized for growth of *S. mutans.*

Recombinant DNA methodology and plasmids. Plasmid pAX1CE was isolated from *S. mutans* pTV21Δ2TetM-convertant AX1C as described previously (17). To recover a larger fragment of *S. mutans* chromosomal DNA flanking the *icd*::Tn*917* insertion, AX1 chromosomal DNA was digested to completion with *Eco*RI and ligated to *Eco*RI-digested and dephosphorylated pUC18 (Pharmacia, Uppsala, Sweden). This ligation reaction was used to transform *E. coli* MC1061, and erythromycin- and ampicillin-resistant transformants were isolated and found to contain pUC18 harboring a 8.7-kb *Eco*RI insert. This plasmid was designated pUC-AX1.

To clone the wild-type *icd* gene, an *S. mutans* genomic library was constructed by ligation of *Eco*RI-digested *S. mutans* JH1005 DNA to *Eco*RI-digested and dephosphorylated plasmid pUC18. One thousand ampicillin-resistant *E. coli* EB106 transformants were screened for the ability to grow on M63 glucose plates in the absence of glutamate. Two glutamate prototrophic clones were isolated, and plasmid pJG400, containing a 3.5-kb *Eco*RI insert, was isolated from one of these clones.

E. coli cells were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.) by the method of Dower et al. (12). To confirm that the Tn*917* insertion was responsible for the glutamate-requiring phenotype of AX1, *S. mutans* NG-8 was transformed with AX1 chromosomal DNA, and erythromycin-resistant, auxotrophic transformants were recovered as described previously (17).

Plasmids were isolated for rapid screening by a boiling technique (20) and purified for DNA sequencing by a polyethylene glycol precipitation procedure (25). Agarose gel electrophoresis was performed as described by Maniatis et al. (25). Southern hybridizations were performed with the Photogene nucleic acid detection system (Gibco/BRL, Bethesda, Md.). Biotin-labelled DNA probes were prepared with the Bio-Nick labelling kit (Gibco/BRL).

DNA sequencing and analysis. Nucleotide sequencing was carried out at the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research. Sequencing was accomplished with *Taq* DiDeoxy terminators and the DyePrimer cycling sequence protocol developed by Applied Biosystems with fluorescently labeled dideoxynucleotides and primers, respectively. Labelled extension products were analyzed on a Biosystems 373A DNA sequencer. To obtain overlapping sequence on both DNA strands, we used 17-mer synthetic oligonucleotide primers obtained from the custom DNA synthesis facilities of National Biosciences, Inc. (Plymouth, Minn.). Sequence analyses were carried out with the Macintosh program MacVector V3.5 (Kodak, Inc., Rochester, N.Y.) and programs BLASTP and BLASTX (NCBI Labs, Los Alamos, N.Mex.). Alignment of deduced amino acid sequences was performed with CLUSTALW (EMBL, Heidelberg, Germany). Construction of the alignment figure and designation of identical and similar amino acid residues were performed with the Macintosh program SeqVu (Garvin Institute of Medical Research, Sydney, Australia). Terminator-like structures were identified with the program Terminator (Genetics Computer Group package, University of Wisconsin, Madison).

Preparation of cell extracts. *E. coli* and *S. mutans* cell extracts were prepared by sonication essentially as described previously (4) with slight modification. Cells from 20 ml of *E. coli* or 500 ml of *S. mutans* cultures were resuspended in 1 or 2 ml, respectively, of 50 mM Tris-HCl buffer (pH 7.5). Sonication and separation of the extracts from the cell debris and glass beads were performed as described before (4). Extracts that were used to determine the effects of various ions, activators, and inhibitors were spin dialyzed in a Centricon 10 concentrator for 2 h at 5,000 × *g* (Amicon, Beverly, Mass.) and washed with 2 ml of 50 mM Tris-HCl buffer (pH 7.5). Extracts were kept on ice until used in assays performed within 6 h. Protein determination was carried out by the bicinchoninic acid method (36).

Enzyme assays. ICDH activity was assayed by a modification of the method of Kornberg (22). Reaction mixtures were incubated at 37°C and contained 35 mM Tris-HCl buffer (pH 7.5), 5 mM DL-isocitrate, 0.35 mM NAD^+ or $NADP^+$, either 3.5 mM $MgCl_2$ or $MnCl_2$, and between 1 and 20 μg of protein from crude cell extracts in a total volume of 1.0 ml. To test for potential activators or inhibitors of ICDH, reaction mixtures were also supplemented with one of the following at 0.15 mM: AMP, ADP, ATP, PEP, α-ketoglutarate, glyoxylate, oxalacetate, glutamate, or glutamine. The formation of NADH was monitored spectrophotometrically by continuous recording of the change in A_{340} for up to 10 min with a UV-160 recording spectrophotometer (Shimadzu Corp., Kyoto, Japan). One unit of activity was defined as 1 nmol of NADH formed min^{-1} mg of protein^{-1}.

Citrate synthase activity was assayed by the method of Fortnagel and Freese (13). This assay indirectly measures the rate of CoA liberated from acetyl-CoA by citrate synthase. This rate was determined spectrophotometrically by monitoring the change in A_{412}, indicating the splitting of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTN) by free CoA. Reaction mixtures were incubated at 37°C and contained 20 mM Tris-HCl buffer (pH 7.5), 20 mM sodium oxalacetate, 0.5 mM acetyl-CoA, and 10 mM DTN in a total volume of 1.0 ml. One unit of activity was defined as 1 nmol of CoA liberated min^{-1} mg of protein^{-1}.

Enzyme activities are expressed as the mean ± standard error of four measurements from cell extracts prepared from two independent cultures.

Chemicals and enzymes. Restriction enzymes, T4 DNA ligase, and DNA molecular weight standards were from Promega (Madison, Wis.) or New England Biolabs (Beverly, Mass.) and were used as directed by the supplier. All

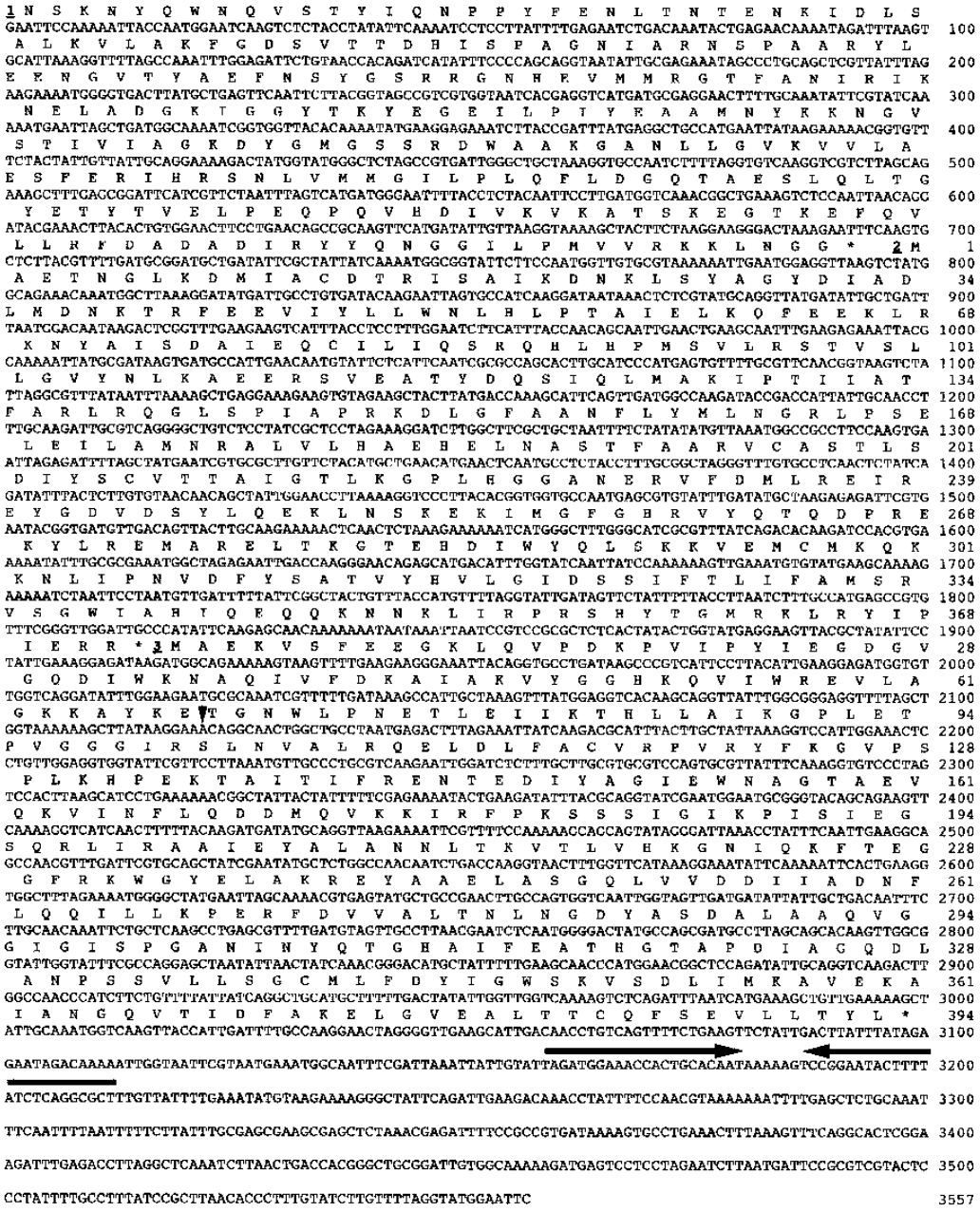

FIG. 2. The entire nucleotide sequence of the *S. mutans* JH1005 chromosomal DNA insert from plasmid pJG400 with the ORFs and their deduced amino acid sequences as illustrated. The 3′ end of the putative *acn* gene (aconitase) begins at the first nucleotide (1) and ends at base 793. The entire genes encoding citrate synthase and ICDH are encoded by nucleotides 798 to 1916 (2) and 1918 to 3099 (3), respectively. The nucleotide and amino acid residue numbers are illustrated at the right of the sequence. The triangle indicates the insertion point of Tn*917* in *S. mutans* AX1. A transcriptional terminator-like structure is illustrated by opposing arrows. The GenBank accession number is U62799.

other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.), Fisher Scientific Limited (Tustin, Calif.), or Difco Laboratories (Detroit, Mich.).

Nucleotide sequence accession number. The GenBank accession number of the *S. mutans 'acn-citZ-icd* gene cluster is U62799.

RESULTS AND DISCUSSION

Growth of *S. mutans* in minimal medium. When laboratory and freshly isolated *S. mutans* strains were tested for their ability to grow on SMM agar containing no amino acids, we found that 14 of 16 strains tested were able to grow anaerobically without an organic nitrogen source (data not shown). The amount of ammonia required to sustain growth of *S. mutans* JH1005 was determined by titration of ammonium sulfate in liquid SMM. Under the conditions tested, this strain required approximately 1 mM ammonium sulfate to achieve a cell density indicative of ammonia saturation. *S. mutans* AX1 (Glu^-) was unable to grow in either solid or liquid SMM unless it was supplemented with 3 mM glutamate.

Genetic characterization of the glutamate-requiring *S. mutans* auxotroph AX1. Southern hybridization of *Eco*RI-digested AX1 chromosomal DNA probed with pTV1-OK revealed a single transposon insertion as indicated by hybridization to a

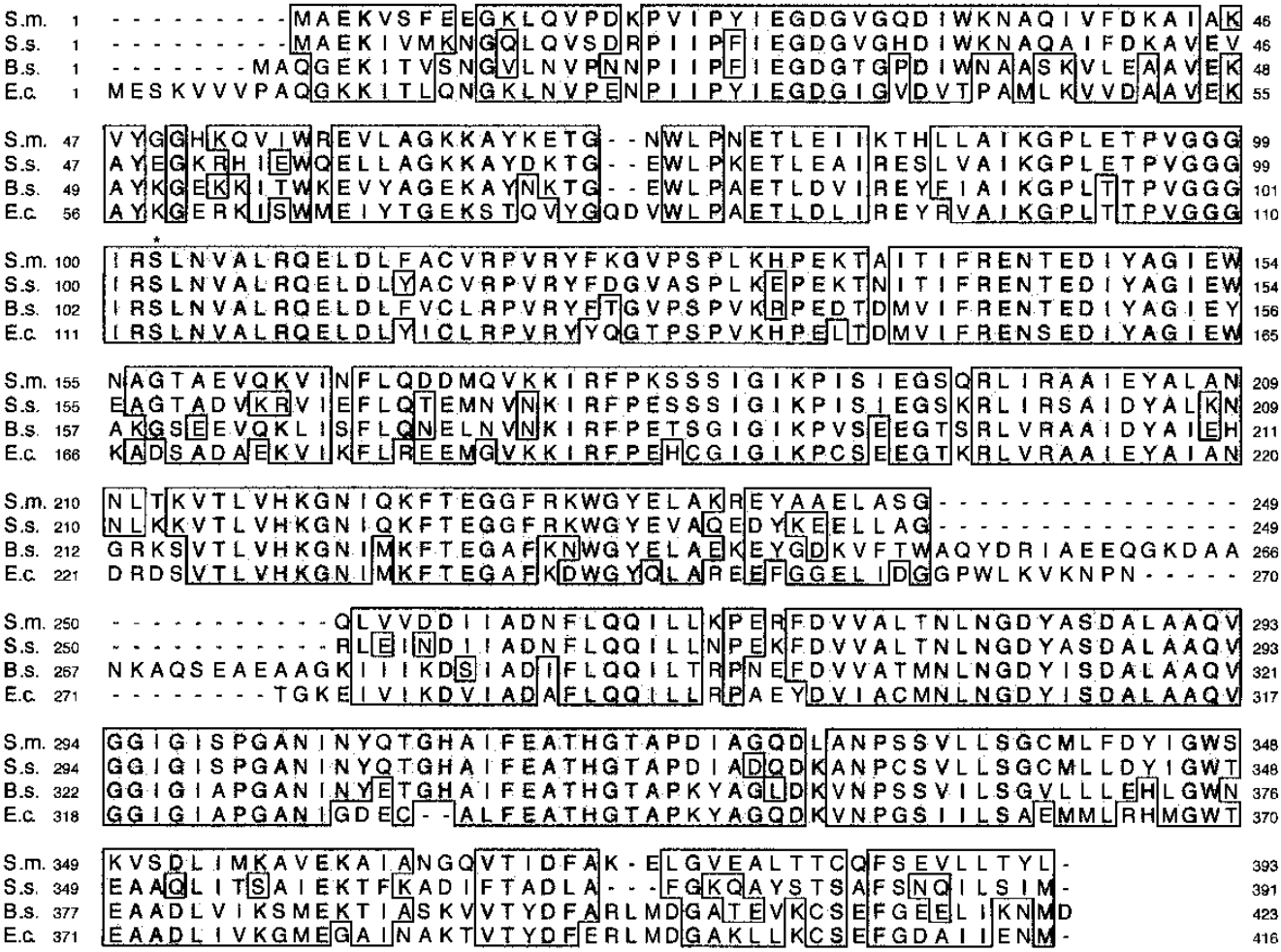

FIG. 3. Alignment of the deduced amino acid sequence of the *S. mutans icd* gene with homologs from other bacterial species. Identical regions are shaded, while regions of homology are outlined. Abbreviations: S.m., *S. mutans*; S.s., *S. salivarius*; B.s., *B. subtilis*; E.c., *E. coli*. The asterisk indicates the conserved serine residue (*E. coli* Ser-113) that is phosphorylated in *E. coli* (24).

single fragment of 8.7 kb (data not shown). To confirm that the Tn*917* insertion was responsible for the glutamate requirement of AX1, chromosomal DNA from this strain was used to transform *S. mutans* NG8. One hundred percent of the erythromycin-resistant NG8 transformants required glutamate for growth, while the parent did not (data not shown).

Preliminary nucleotide sequence analysis of the transposon recovery vector pAX1CE revealed that Tn*917* had inserted into a region of the *S. mutans* JH1005 chromosome that had a high degree of homology to the 3′ end of other bacterial *icd* genes encoding ICDH (17). Further sequence analysis of plasmid pUC-AX1 confirmed that Tn*917* had inserted into a putative *icd* gene 204 nucleotides downstream from the 5′ end of a 1,182-nucleotide open reading frame (ORF).

Isolation and analysis of the intact *S. mutans icd* and *citZ* genes. The intact *S. mutans icd* gene was isolated on a 3.5-kb *Eco*RI fragment harbored on plasmid pJG400 as described in Materials and Methods. This fragment contained the complete *S. mutans icd* gene in an ORF of 1,182 nucleotides encoding a putative ICDH of 393 amino acid residues, with a predicted molecular mass of 43,101 Da (Fig. 2).

A putative terminator-like structure consisting of a region of dyad symmetry had 75% identity to a previously identified *S. mutans* terminator (4). Sequence comparisons of pJG400 with the sequence obtained from the transposon recovery vectors pAX1CE and pUC-AX1 revealed 100% homology in the regions encoding *S. mutans* chromosomal DNA, confirming that pJG400 contained the corresponding wild-type allele from the parent strain JH1005.

Sequence comparison of the deduced *S. mutans* ICDH revealed that it had significant homology to $NADP^+$-dependent dimeric forms of ICDH from other bacteria, including *Bacillus subtilis* (21) and *E. coli* (40), displaying 60 and 62% homology, respectively (Fig. 3). The deduced amino acid sequence of the protein encoded by *S. mutans icd* was most similar (72%) to the ICDH from the closely related oral organism *Streptococcus salivarius* that has been reported to be an NAD^+-dependent enzyme (10). There is very little homology (32%) with sequences recently obtained (30) for the NAD^+-dependent ICDH from the primate *Macaca fascicularis*. A search of the genetic database revealed no greater homology than 32% when comparing the *S. mutans* ICDH with analogous eukaryotic enzymes.

Nucleotide sequence analysis of pJG400 in the region located 5′ from the *S. mutans icd* revealed that the plasmid contained another complete putative ORF which was sepa-

TABLE 1. Enzyme activities in *S. mutans* and *E. coli* cell extracts

Strain	ICDH activity[a]		Citrate synthase activity[b]
	$NADP^+$	NAD^+	
S. mutans			
JH1005			
Grown in SMM	ND[c]	234 ± 60	135 ± 32
Grown in THBYE	ND	123 ± 10	66 ± 13
AX1	ND	ND	—[d]
E. coli			
DH5α	373 ± 17	5 ± 0.7	316 ± 61
EB106(pUC18)	ND	ND	—
EB106(pJG400)	ND	1,860 ± 28	—
W620(pUC18)	—	—	16 ± 2
W620(pJG400)	—	—	404 ± 107

[a] One unit of activity was defined as 1 nmol of NADH formed min^{-1} mg of $protein^{-1}$.
[b] One unit of activity was defined as 1 nmol of CoA liberated min^{-1} mg of $protein^{-1}$.
[c] ND, not detected.
[d] —, not done.

rated from the *icd* gene by a single base pair. This ORF was 1,119 nucleotides in length and encoded a deduced protein of 372 amino acids with a predicted molecular mass of 42,759 Da. This deduced protein sequence had a high degree of homology with citrate synthases (EC 4.1.3.7) from other bacteria. A comparison of the deduced amino acid sequence of the protein encoded by the *S. mutans citZ* gene was made with the amino acid sequences of the homologs from *Bacillus subtilis* (citrate synthase II, *citZ*) (21), *Mycobacterium smegmatis* (8), and *Pyrococcus furiosus* (28). The degrees of homology were 53, 43, and 43%, respectively. The *S. mutans citZ* gene was most similar to *citZ*, which encodes citrate synthase II from *B. subtilis*, an organism that has at least two citrate synthase genes, *citA* and *citZ*, which have 42% identity at the amino acid level (21). We did not investigate the possibility of *S. mutans* possessing more than one form of the enzyme.

The plasmid pJG400 also contained a third putative ORF which, by similar analysis, appeared to encode the 3′ end of the gene coding for aconitase (Fig. 2), the enzyme that converts citrate to isocitrate. Sequence analysis of the pJG400 *Eco*RI insert revealed no promoter-like structures located 5′ from either the *icd* or *citZ* genes and contained only the 3′ end of the *acn* gene. This suggested that transcription of *icd* and *citZ* in *E. coli* was likely initiated from the *lacZ* promoter of the plasmid, located 5′ proximal to the insert.

The intact *S. mutans* aconitase gene has not yet been cloned nor its enzymatic activity detected, although its presence is strongly suggested from the partial sequence and its biological role in the intermediate step between citrate synthase and ICDH. Investigation into the role of this enzyme will warrant further study due to its inherent instability (11) and complex regulation (14, 15).

Activities of the native and cloned *S. mutans icd* and *citZ* gene products. ICDH activities measured in *S. mutans* and *E. coli* crude cell extracts are described in Table 1. The ICDH activity of strain DH5α was included as an indication of wild-type *E. coli* activities. No ICDH activity was detected in either *S. mutans* AX1 or the *icd E. coli* control strain EB106 harboring pUC18. EB106 containing the *S. mutans icd* gene on pJG400 expressed more than 10 times the activity of the *S. mutans* wild-type strain under the same assay conditions.

ICDH activity in *S. mutans* and in the *E. coli* mutant complemented with pJG400 was completely dependent on NAD^+, while the addition of $NADP^+$ was unable to facilitate activity (Table 1). The wild-type *E. coli* DH5α ICDH, however, required $NADP^+$. This was an interesting observation, since NAD^+-dependent ICDHs are more commonly associated with eucaryotic organisms (33). When our data are considered with data obtained by Desgagnes et al. (10), it appears that the streptococcal ICDHs may be unique among bacteria in regard to their NAD^+ dependency.

The eucaryotic NAD^+-dependent ICDH (EC 1.1.1.41) is often regulated by AMP or ADP (22, 33). The compounds AMP, ADP, ATP, PEP, α-ketoglutarate, glyoxylate, oxalacetate, glutamate, and glutamine, however, had no significant effect on ICDH activity in the wild-type *S. mutans* extracts (data not shown), suggesting that ICDH may not be regulated at the enzyme level in *S. mutans*.

The *S. mutans* ICDH also required Mg^{2+} or Mn^{2+} to function, since dialyzed extracts without these ions had no detectable activity (data not shown). Mn^{2+} was the preferred cation since activity in the *S. mutans* extract was about twofold higher with Mn^{2+} than with Mg^{2+} (data not shown). The ICDH data presented in Table 1 were obtained with Mn^{2+} as a cofactor.

Citrate synthase activity was detected in the *S. mutans* parent strain JH1005 and in the pJG400-complemented *E. coli gltA* mutant W620 (Table 1). Some activity was also detectable in *E. coli* W620 containing pUC18 but at an insignificant level when compared with activities in DH5α and W620 harboring pJG400 (Table 1). *E. coli* W620 harboring the *S. mutans citZ* gene on pJG400 had an 80-fold increase in activity over the background observed with the pUC18-containing host and had comparable activity to *E. coli* DH5α.

There was approximately a twofold increase in *S. mutans* JH1005 ICDH and citrate synthase activities when the cells were grown in minimal medium (SMM) as compared with cells grown in a rich medium (THBYE) (Table 1). There were no apparent differences when cells were harvested at the exponential or the stationary phase (data not shown).

In aerobic and facultative anaerobic bacteria with a complete TCA cycle, this extensively studied pathway is involved in the generation of energy and functions in the biosynthesis of amino acids (19, 31). Some lactobacilli have an incomplete TCA cycle, presumably used for synthesis of succinate, and are auxotrophic for glutamate since they lack a functional ICDH (27). Other organisms, including thiobacilli (35), clostridia (37), and cyanobacteria (29, 32), also contain a partial TCA cycle utilized solely for biosynthetic functions. These organisms lack one or more of the enzymes of the TCA cycle, with α-ketoglutarate dehydrogenase being absent in all of the cases mentioned above. The presence of α-ketoglutarate dehydrogenase or other TCA cycle enzymes in *S. mutans* was not determined in this study, but a similar truncated TCA cycle seems likely to exist in this organism.

The ICDH of *E. coli* is a key regulatory enzyme which is inactivated by phosphorylation at serine-113 to direct the flow of isocitrate through the glyoxylate shunt to bypass the CO_2-generating steps of the TCA cycle (24). This latter process is necessary for anapleurotic reactions during growth on two-carbon compounds such as acetate. An analogous serine residue is also present in the *S. mutans* ICDH in a region that is highly conserved among the compared sequences (Fig. 3). In *E. coli*, this region has been attributed to be part of the enzymatic active site involved in the direct binding of isocitrate to the enzyme (24). The lack of a complete TCA cycle and glyoxylate bypass in *S. mutans* suggests that a regulatory role for this enzymatic site in the streptococcal ICDH is unlikely.

The mechanism of glutamate biosynthesis in *S. mutans* is of interest since it may be an essential process used by the organ-

ism to survive under the conditions it encounters in dental plaque. In the lower levels of plaque, this system would function under anaerobic conditions, where free amino acids are likely to be limiting due to their consumption by the competing microflora inhabiting the more-nutrient-abundant upper layers of the biofilm matrix. In the lower levels of plaque, simple nitrogenous compounds such as ammonia, generated from the hydrolysis of salivary urea by the activity of other members of the community, are possibly the sole source of nitrogen available to *S. mutans*. Further investigations will examine the mechanisms that *S. mutans* utilizes under these conditions, with the ability of this organism to contend with nitrogen starvation likely playing a key role in its survival.

ACKNOWLEDGMENTS

We acknowledge Michel Frenette from Laval University, Quebec, Canada, and Henry C. Reeves, Arizona State University, Tempe, for helpful discussions and insightful information, Paula Crowley of the University of Florida for expert technical advice, and Jeffrey D. Hillman and Jeannine Brady of the University of Florida for critical review of the manuscript.

This research was supported by grant DE 08007 from the National Institute for Dental Research.

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