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Nucleotide Sequence of a *Porphyromonas gingivalis* Gene Encoding a Surface-Associated Glutamate Dehydrogenase and Construction of a Glutamate Dehydrogenase-Deficient Isogenic Mutant

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The nucleotide sequence for a surface-associated protein (A. Joe, A. Yamamoto, and B. C. McBride, Infect. Immun. 61:3294–3303, 1993) of *Porphyromonas gingivalis* was determined. The structural gene comprises 1,338 bp and codes for a protein of 445 amino acids. The deduced molecular weight of the protein is 49,243. A data base search for homologous proteins revealed significant sequence similarity to the subunit protein of glutamate dehydrogenases (GDHs) isolated from various sources. This protein, which was previously labelled PgAg1, will now be called GDH. Recombinant GDH was purified to homogeneity, and native GDH was partially purified from *P. gingivalis*. Both preparations exhibited NAD-dependent GDH activity. Intact *P. gingivalis* and an extract of cell surface components also demonstrated NAD-dependent GDH activity. To help elucidate the role of this protein, an isogenic mutant of *P. gingivalis* lacking the GDH protein was generated by deletion disruption. Biological characterization of the mutant strain, *P. gingivalis* E51, demonstrated complete loss of GDH activity. Immunogold bead labelling of intact cells showed that GDH was no longer present on the surface of the bacterial cell. The GDH-negative mutant displayed impaired cell growth, as demonstrated by an increased generation time and an inability to grow to the same cell density as the parent.

A large body of evidence from microbiological (6, 40, 44) and serological (13, 43) studies implicates *Porphyromonas* (*Bacteroides*) *gingivalis* as a periodontopathogen. Studies of the microbial population of healthy gingival sulci and periodontal pockets (44) show the presence of *P. gingivalis* in samples taken from periodontal pockets of patients with periodontitis. This organism was not detected in samples taken from healthy sites. Most periodontal patients mount a humoral immune response towards *P. gingivalis* during the course of a periodontal infection (13, 43). Immunological studies with sera from patients with periodontal disease have identified immunodominant outer membrane-associated molecules with sizes of 75 (45), 53 (23), and 47 (8) kDa. Curtis et al. (8) found a correlation between the presence of antibody to the 47-kDa protein and active periodontitis.

We have previously reported the cloning and expression of a 51-kDa surface-associated protein (previously designated PgAg1) of *P. gingivalis* (22). It was of interest that the NH_2-terminal amino acid sequence of this recombinant protein was identical to the NH_2-terminal amino acid sequence of the 47-kDa immunodominant antigen identified by Curtis and coworkers (8).

In this report, we present the nucleotide sequence of the gene and show that it codes for a glutamate dehydrogenase (GDH). To help elucidate the role of GDH, an isogenic mutant of *P. gingivalis* lacking the enzyme was generated.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. gingivalis* ATCC 33277 and E51 (generated during this study) were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml). Laked blood and agar were added to final concentrations of 5% (vol/vol) and 1.5% (wt/vol), respectively, to prepare BHI-blood agar plates. Erythromycin and gentamicin at final concentrations of 10 and 200 μg/ml, respectively, were used in BHI medium when required. Growth was at 37°C in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) containing an atmosphere of N_2-H_2-CO_2 (85:10:5).

Escherichia coli JM83, XL1, and J53 were used as hosts for plasmid DNA. Cultures were grown in Luria-Bertani broth (31) at 37°C. Ampicillin, kanamycin, and trimethoprim were used at final concentrations of 100, 50 to 75, and 200 μg/ml, respectively, in the medium when required.

Surface extract of *P. gingivalis*. An extract containing cell surface-associated components of *P. gingivalis* was obtained as follows. Cells from a late-exponential-phase culture of *P. gingivalis* ATCC 33277 were harvested and washed twice with prereduced phosphate-buffered saline (PBS) (pH 7.2). The cells were resuspended in 30 mM prereduced Tris-HCl (pH 7.4) containing *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK) and phenylmethylsulfonyl fluoride at concentrations of 10 and 0.2 mM, respectively. The cell suspension was placed inside an anaerobic chamber (Coy Manufacturing) containing an atmosphere of N_2-H_2-CO_2 (85:10:5), and a magnetic stir bar was added. The cells were stirred for 1 h. The cell suspension was centrifuged (10,000 × *g*, 15 min, 4°C) to obtain a surface extract (the supernatant) and a cell pellet. The pellet was washed twice with PBS (pH 7.2), and the cells were resuspended in 30 mM Tris-HCl (pH 7.4) containing TLCK and phenylmethylsulfonyl fluoride. The surface extract was centrifuged at low speed to remove residual cells and then was subjected to high-speed centrifugation (100,000 × *g*, 1 h, 4°C).

The supernatant was collected as the soluble surface fraction, and the pellet was recovered as the insoluble surface fraction. The soluble surface fraction was recentrifuged at high speed to remove any residual insoluble components. The insoluble surface fraction was washed with 30 mM Tris-HCl (pH 7.4), and the pellet was resuspended in 30 mM Tris-HCl (pH 7.4) containing TLCK.

Electrophoretic techniques. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with the buffer system of Laemmli (24). With the exception of whole cells of *P. gingivalis*, samples were prepared for SDS-PAGE by the addition of sample buffer (62.5 mM Tris-HCl [pH 6.8], 5% [vol/vol] β-mercaptoethanol, 1% [wt/vol] SDS, 10% [vol/vol] glycerol, 0.012% [wt/vol] bromophenol blue) followed by heating in boiling water for 10 min. Whole-cell lysate samples of *P. gingivalis* were prepared as follows. Cells were harvested, washed twice with 0.85% (wt/vol) NaCl, and incubated with 20 mM TLCK for 10 min on ice. Sample buffer was added, and the preparation was placed in boiling water for 10 min. Two sets of molecular weight standards were used. Set 1 contained bovine serum albumin (BSA) (66,200), ovalbumin (45,000), gluteraldehyde-3-phosphate dehydrogenase (36,000), trypsinogen (24,000), β-lactoglobulin (18,400), and lysozyme (14,300). Set 2 contained the prestained proteins myosin (H chain) (224,000), phosphorylase *b* (109,000), BSA (72,000), ovalbumin (45,800), and carbonic anhydrase (28,500).

Discontinuous nondenaturing PAGE was carried out in the same manner as SDS-PAGE, except reagents were prepared without SDS. Samples for nondenaturing PAGE were prepared by adding sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 0.012% bromophenol blue); heat was not applied.

Following electrophoresis, gels were treated in one of three ways. (i) They were stained for proteins with silver nitrate (28), (ii) they were transferred to nitrocellulose for Western blotting (immunoblotting) (41), or (iii) they were processed for detection of GDH activity (GDH zymogram).

Antibodies and Western blot immunoassays. An affinity-purified polyclonal antibody preparation specific for the recombinant protein rGDH (αrGDH) was prepared as described previously (22). Immobilized proteins on Western blots which reacted with specific antibodies were detected by incubation with goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Bethesda Research Laboratories Inc., Gaithersburg, Md.) followed by incubation with a dye solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Purification of GDH. Cells from overnight-grown cultures of *E. coli* BA3 were harvested and washed once with 20 mM Tris-HCl (pH 7.4). The cells were resuspended in 20 mM Tris-HCl (pH 7.4) containing 10 mM $MgCl_2$ and 20 μg (each) of RNase A and DNase I per ml and were passed two times through a cold Aminco French pressure cell at 20,000 lb/in^2. The broken cell suspension was subjected to high-speed centrifugation (100,000 × *g*, 60 min, 4°C), and the supernatant containing soluble proteins was recovered for further processing.

The supernatant was concentrated by lyophilization, dialyzed against 20 mM Tris-HCl (pH 7.4), and then applied to a MonoQ anion-exchange column (fast protein liquid chromatography [FPLC] system; Pharmacia, Uppsala, Sweden) in which proteins were eluted in a step gradient of 0.1 M NaCl, 0.2 M NaCl, and 1.0 M NaCl in 20 mM Tris-HCl (pH 7.4). Fractions containing protein were analyzed by SDS-PAGE for the presence and purity of rGDH.

Fractions containing rGDH were applied to a Superose 12 gel filtration column (Pharmacia FPLC system), and proteins were eluted with a buffer of 20 mM Tris-HCl–100 mM NaCl (pH 7.4).

The native GDH protein was partially purified from *P. gingivalis* ATCC 33277 by a modification of the procedure given by Curtis et al. (8). Briefly, *P. gingivalis* cells were harvested and incubated on ice with TLCK and phenylmethylsulfonyl fluoride at concentrations of 20 and 0.2 mM, respectively. EDTA was added to a final concentration of 100 mM, and the cell suspension was incubated at 60°C for 30 min and then was chilled on ice for 10 min.

$MgCl_2$ and RNase A and DNase I were added to final concentrations of 10 mM and 20 μg/ml (each), respectively, and the cell suspension was passed five times through a cold Aminco French pressure cell at 20,000 lb/in^2. The broken cell suspension was subjected to high-speed centrifugation (100,000 × *g*, 60 min, 4°C) and the supernatant containing GDH was recovered for protein purification. Column fractionation was performed as described for rGDH.

Protein concentration was determined with the Bradford reagent (Bio-Rad Laboratories, Hercules, Calif.), with BSA as the standard.

General recombinant DNA methods. Isolation of plasmid DNA, restriction endonuclease digestions, agarose gel electrophoresis, and ligations were performed according to standard procedures (31). Purification of DNA fragments was by excision from agarose gels and phenol extraction, as described by Silhavy et al. (36). Transformations were conducted by the method of Chung et al. (7). DNA fragments in a λ-*Hin*dIII preparation (Bethesda Research Laboratories, Inc.) were used as DNA molecular weight standards. Southern blot hybridization analysis was performed with the BluGENE nonradioactive nucleic acid detection system (Bethesda Research Laboratories, Inc.).

Nucleotide sequencing and sequence analysis. The nucleotide sequence was determined by an automated fluorescent procedure based on the Sanger dideoxy-chain termination method (32). The *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems Inc., Foster City, Calif.) was used per the manufacturer's recommendations. A stock preparation of double-stranded pBA3-7 plasmid (2-kb insert DNA) was used as template DNA.

Nucleotide sequence data were obtained by stepwise sequencing. Initially, universal and reverse primers of pUC18 were used. As new DNA sequence data became available, customized synthetic primers (24-mer) were utilized (the University of British Columbia Oligonucleotide Synthesis Laboratory). The sequencing reaction products were analyzed with the Applied Biosystems model 373A DNA sequencer.

Both strands of DNA were completely sequenced. The nucleotide sequence was analyzed with the DNA Strider (version 1.0) (Service de Biochimie, Department de Biologie, Institut de Recherche Fondamentale Commissariat a l'Energie Atomique, Saclay, France) and PC/Gene (release 6.5) (IntelliGenetics Inc., Mountain View, Calif.) programs. The SWISS-PROT, PIR, and GenBank data bases were searched for homologous amino acid sequences with the BLAST (2) network service at the National Center for Biotechnology Information, Bethesda, Md.

GDH assay. GDH activity was measured spectrophotometrically as the reduction of α-ketoglutarate to glutamate in the presence of NAD(P)H (20) by monitoring the oxidation of NAD(P)H to NAD(P) at 340 nm.

One unit of GDH activity was defined as the amount of enzyme required to reduce 1.0 μmol of α-ketoglutarate to L-glutamate per min at 25°C and pH 8.1. Bovine GDH of

known specific activity (Sigma Chemical Co., St. Louis, Mo.) was used as a positive control sample in all assays.

GDH activity in the reverse reaction was also examined by measuring the reduction of NAD(P) during the oxidation of glutamate to α-ketoglutarate.

GDH zymogram. Test samples electrophoresed in nondenaturing polyacrylamide gels were stained for GDH activity by incubation in a solution of 88 mM Tris-HCl (pH 8.5) containing 113 mM glutamate, 0.7 mg of NAD or NADP per ml, 23 μg of phenazine methosulfate per ml, and 0.4 mg of nitroblue tetrazolium dye per ml (14). Enzyme activity appeared as a dark purple band.

Immunogold labelling of *P. gingivalis*. For immunogold labelling of intact cells, *P. gingivalis* was harvested from an early-log-phase culture and resuspended in PBS (pH 7.2) to an optical density at 660 nm of 0.85. The cell suspension was placed on 200-mesh nickel grids coated with Collodion. Excess liquid was removed, and the cell-labelling assay was performed by floating grids successively on the surface of a drop (each) of 3% (wt/vol) BSA in Tris-buffered saline (20 mM Tris, 0.5 M NaCl [pH 7.5]), affinity-purified antibodies diluted in 1% BSA–Tris-buffered saline, and goat anti-rabbit immunoglobulin G–10-nm-diameter gold bead conjugate (Sigma Chemical Co.) diluted in 1% BSA–Tris-buffered saline. Between each solution, grids were washed thoroughly with Tris-buffered saline. Cells were negatively stained with 0.5% (wt/vol) ammonium molybdate and viewed with a Philips 300 transmission electron microscope.

Generation of an isogenic mutant. A GDH-negative isogenic mutant of *P. gingivalis* ATCC 33277 was generated as follows. Plasmid pBA3-3 was digested with *Acc*I, and a 5.5-kb digestion product was recovered. The ends of the DNA molecules were filled in by treatment with Klenow fragment in the presence of deoxynucleoside triphosphates; 5′-terminal phosphates were removed with calf intestinal alkaline phosphatase. The 5.5-kb fragment was ligated to a 3.4-kb *Eco*RI-*Cla*I DNA fragment isolated from plasmid pNJR5. The DNA construct obtained was designated pBA3-10 and was maintained in *E. coli* by ampicillin selection. Plasmid pNJR5, a *Bacteroides-E. coli* shuttle vector (35), was kindly supplied by Nadja Shoemaker and Abigail Salyers (University of Illinois, Urbana). The plasmid pBA3-10 contained a modified *gdh* gene. An internal 400-bp portion of *gdh* was replaced with the *Bacteroides* erythromycin resistance (Em^r) gene from pNJR5.

A 9.7-kb *Bam*HI-*Cla*I fragment was isolated from pNJR5 and treated with calf intestinal alkaline phosphatase to remove 5′-terminal phosphates. The treated 9.7-kb fragment was ligated to a 5.7-kb *Bam*HI-*Cla*I fragment isolated from pBA3-10. The DNA construct obtained was designated pJOE1 and was maintained in *E. coli* by kanamycin selection. Plasmid pJOE1 constituted an *E. coli-Bacteroides* suicide shuttle vector carrying a mutated allele of *gdh*.

For mobilization of pJOE1 from *E. coli* to *P. gingivalis*, the helper plasmid R751 in *E. coli* J53 was introduced into *E. coli* XL1/pJOE1 by *E. coli-E. coli* mating. Transconjugants carrying both plasmids were isolated on medium containing 10 μg of kanamycin per ml (to select for pJOE1) and 200 μg of trimethoprim per ml (to select for R751).

The deletion-disrupted *gdh* gene was introduced into *P. gingivalis* ATCC 33277 by conjugal mating with *E. coli* donor cells as follows. Cultures of *E. coli* XL1/R751,pJOE1 and *P. gingivalis* ATCC 33277 were grown to early exponential phase (optical density at 660 nm of 0.2). A volume of 0.2 ml of *E. coli* XL1/R751,pJOE1 and 1 ml of *P. gingivalis* was mixed in a sterile microcentrifuge tube. The cells were harvested and resuspended in 0.2 ml of prereduced BHI broth. The cell mixture was transferred to a sterile cellulose membrane placed on prereduced BHI-blood agar. The plate was incubated anaerobically at 37°C for 48 h, the membrane was lifted from the agar, and the cells were shaken off into 3 ml of prereduced BHI broth. Aliquots of 0.2 ml of the cell suspension were plated onto prereduced BHI-blood agar containing 10 μg of erythromycin per ml and 200 μg of gentamicin per ml (primary selection plates). Erythromycin was used to select for *P. gingivalis* cells containing a chromosomal Em^r gene which had been introduced on pJOE1. Gentamicin was included in the medium to inhibit growth of *E. coli* donor cells. The plates were incubated anaerobically at 37°C for 4 weeks.

Colonies appearing on primary selection plates were passaged twice on BHI-blood agar supplemented with erythromycin and gentamicin. Those strains which were viable on the third selection were inoculated into BHI broth containing 10 μg of erythromycin per ml.

Analysis of fatty acids. Volatile and derivatized nonvolatile fatty acids produced by *P. gingivalis* were analyzed by gas chromatography. To obtain a profile of volatile fatty acids, culture supernatant was acidified with H_3PO_4 and then was analyzed in a Perkin-Elmer (Norwalk, Conn.) Sigma 3B dual flame ionization detection chromatograph equipped with a hydrogen flame ionization detector. Samples were separated on a 60/80 Carbopack C–0.3% Carbowax 20M–0.1% H_3PO_4 column (Supelco Canada, Oakville, Ontario, Canada). Helium was used as the carrier gas. Methyl ester derivatives of nonvolatile fatty acids were generated by treating culture supernatant with methanol and H_2SO_4 at 60°C for 30 min. Esters were extracted into chloroform and chromatographed on 15% SP-1220–1% H_3PO_4 on a 100/120 Chromosorb W AW column (Supelco Canada).

Nucleotide sequence accession number. The nucleotide sequence for the *gdh* gene has been entered into several data banks (GenBank, EMBL Data Library, and DNA Data Bank of Japan) under the title "*P. gingivalis gdh* gene," accession no. L07290.

RESULTS

Localization of the *gdh* gene. A series of deletion subclones (Fig. 1) were generated in order to define the location of the *gdh* (previously *pgag*1) structural gene and the promoter elements for transcriptional initiation. As described in a previous report (22), rGDH is highly expressed from both the original recombinant plasmid pBA3 and the plasmid pBA3R, which contains the 3.7-kb insert DNA in the reverse orientation. This indicated that the *gdh* gene was expressed from promoter elements located within the cloned DNA fragment.

E. coli cells carrying the plasmid pBA3-2 expressed a 23-kDa truncated rGDH protein as detected by Western blot immunoassay with αrGDH. This suggested that transcription of the *gdh* gene was initiated from the *Cla*I end of the 3.7-kb *Pst*I-*Pst*I insert in the original recombinant plasmid pBA3. The *Cla*I end of the cloned fragment was therefore defined as the proximal end of the DNA insert with regard to the cloned promoter. In the original recombinant clone *E. coli* BA3, the direction of transcription of the *gdh* gene from the cloned promoter is opposite to the direction of transcription from the *lacZ′* promoter carried on pUC18.

The subclone containing pBA3-5 showed that removal of a 500-bp fragment from the proximal end of the insert DNA by *Cla*I cleavage did not have any effect on the high level of expression of the recombinant protein. However, removal of an additional 300 bp from the proximal end by *Eco*RI cleavage (to generate pBA3R-6) resulted in the loss of expression of

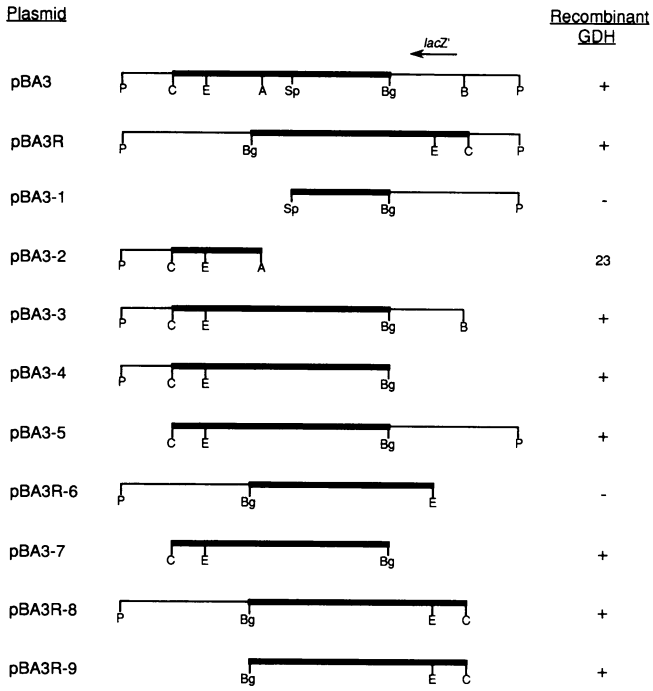

FIG. 1. Partial restriction map and characteristics of deletion subclones derived from pBA3. Clones were analyzed for expression of recombinant protein by SDS-PAGE. A high level of 48-kDa rGDH is denoted by a plus sign. Subclones in which rGDH was not clearly identified in a protein profile were analyzed by Western blot immunoassay with αrGDH. Clones with no detectable rGDH are denoted by a minus sign. A truncated 23-kDa rGDH detected by Western blot immunoassay analysis is represented by 23. P, *Pst*I; B, *Bam*HI; Bg, *Bgl*II; Sp, *Sph*I; A, *Acc*I; E, *Eco*RI; C, *Cla*I.

rGDH, suggesting that the promoter elements and the translational ATG start signal for the *gdh* gene were located within the 300-bp *Cla*I-*Eco*RI region of the insert DNA fragment. If the cloned DNA in pBA3R-6 contained the *gdh* structural gene complete with its translational start signal, one would have expected expression of the recombinant protein from the *lacZ'* promoter.

High-level expression of rGDH in subclones carrying the deletion plasmids pBA3-7 and pBA3R-9 indicated that both the cloned promoter elements and the *gdh* structural gene were contained within a 2-kb *Cla*I-*Bgl*II region.

DNA sequence of *gdh*. The nucleotide sequence of the 2-kb *Cla*I-*Bgl*II *P. gingivalis* DNA fragment is given in Fig. 2. The largest translational open reading frame (ORF), comprising 1,338 bp, extends from the ATG start codon at nucleotide 226 to the ochre stop codon ending at nucleotide 1563. This ORF codes for a protein of 445 amino acids with a molecular weight of 49,243. This is in close agreement with the apparent molecular mass (51 kDa) of the native GDH molecule. The first 29 residues of the deduced amino acid sequence from this ORF are identical to the NH_2-terminal amino acid sequence of GDH determined previously by protein sequencing (22). It was evident that this ORF constituted the structural gene for GDH.

The PC/Gene nucleic acid analysis computer program was used to identify potential promoter sequences and a ribosomal binding site (rbs). Putative −35 and −10 promoter regions were located on the basis of homology with *E. coli* promoter consensus sequences. A putative rbs was identified by homology with other prokaryotic Shine-Dalgarno rbs sequences. A search for an NH_2-terminal signal sequence failed to identify a coding region which could potentially translate into a leader peptide.

A hydropathy plot of the translated protein obtained by Kyte-Doolittle analysis showed that the final 20 residues of the COOH terminal of the protein form a hydrophobic region.

A scan of the SWISS-PROT, PIR, and GenBank data bases with the BLAST search algorithm revealed significant sequence similarity between GDH and the subunit protein of GDH enzymes isolated from various sources (Table 1). The active site of GDH appears to involve two lysine residues which are highly conserved in GDH enzymes. These residues are lysines 27 and 126 in the bovine GDH, lysines 14 and 113 in the *Neurospora crassa* NAD-dependent GDH (17), and lysines 25 and 112 in the *P. gingivalis* GDH protein.

Purification of GDH. Pure recombinant GDH was obtained as shown in Fig. 3. Two forms of GDH with molecular masses of 48 and 51 kDa were identified previously (22). In the purification of recombinant protein from BA3, the 51-kDa form of rGDH disappeared, resulting in samples containing the 48-kDa species and a degradation product of 25 kDa. The 25-kDa degradation product is frequently detected in BA3 samples probed with αrGDH in a Western blot immunoassay (22).

Native GDH containing a single contaminating protein was obtained by following the chromatographic procedure used for the recombinant GDH. As observed for the recombinant protein, the 51-kDa form of the native protein was lost during the course of purification. The 51-kDa form was predominant in the starting material, but by the last chromatographic step, it had been degraded to the 48-kDa form.

GDH activity of recombinant and native GDH. The cloned protein was identified as a GDH enzyme by amino acid homology. It was of interest to analyze the recombinant and native GDH proteins for biological activity.

Fractions containing rGDH were tested by a spectrophotometric assay for GDH activity with α-ketoglutarate as the enzyme substrate. When NADH was supplied as the cofactor, the purified recombinant protein exhibited a high level of GDH activity (Table 2). The purified rGDH protein did not show a significant level of GDH activity in the presence of NADPH. When L-glutamate was provided as the enzyme substrate, all samples (including the bovine GDH control) exhibited significantly slower reactivity. Subsequent spectrophotometric assays of GDH activity were therefore conducted with α-ketoglutarate and NADH.

GDH zymograms with L-glutamate as substrate were also used to characterize the biological activity of the recombinant and native GDH molecules. Soluble fractions from *E. coli* JM83/pUC18 and BA3, purified rGDH, and partially purified native GDH preparations were electrophoresed in a nondenaturing polyacrylamide gel. A protein profile obtained by staining the gel with silver nitrate showed that the recombinant protein was clearly defined among the *E. coli* proteins (Fig. 4A, lane 2). A GDH zymogram containing the *E. coli* and GDH samples with NAD as a cofactor showed a single band signifying the *P. gingivalis* GDH in samples containing rGDH or native GDH (Fig. 4B). When NADP was supplied as cofactor, no apparent GDH activity was detected in any of the samples containing *P. gingivalis* rGDH or native GDH (Fig. 4C). In the protein profile, the migration distance of nondenatured recombinant and native GDH corresponded to the migration distance of the NAD-dependent GDH enzymatic activity detected in the GDH zymogram. *E. coli* synthesizes a single NADP-dependent GDH (17). This enzyme was detected in the

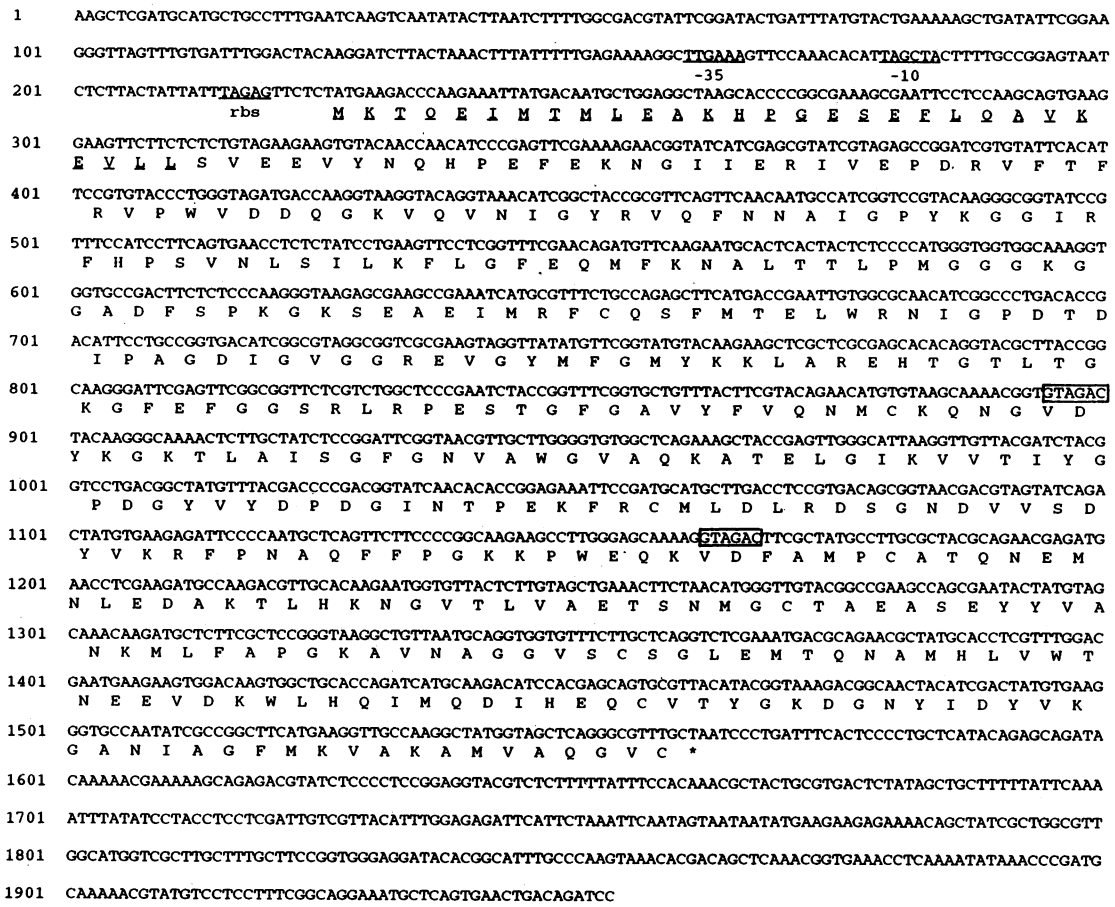

FIG. 2. Nucleotide sequence of the *Cla*I-*Bgl*II fragment containing the *P. gingivalis gdh* gene and the translated amino acid sequence of the coding region. Residues confirmed by NH_2-terminal amino acid sequencing of the GDH protein are underlined. A putative rbs and −35 and −10 promoter regions upstream of the *gdh* ORF are indicated. The recognition sequence (boxed) for the restriction enzyme *Acc*I is shown.

E. coli JM83/pUC18 sample when assayed in the presence of NADP (Fig. 4C, lane 1). Surprisingly, the *E. coli* GDH was not detected in the clone BA3 sample (Fig. 4C, lane 2) containing the rGDH.

Cell surface-associated GDH activity in *P. gingivalis*. Previous analysis of *P. gingivalis* cells by immunogold electron microscopy demonstrated that the GDH protein is associated with the outer surface of the bacterial cell (22). Of interest was whether the surface-associated form of GDH was enzymatically active. Loosely associated surface components of *P. gingivalis* were removed by vigorously stirring the bacterial cells. The following *P. gingivalis* samples were examined for the presence of GDH and GDH activity: whole *P. gingivalis* cells, *P. gingivalis* cells postagitation, a surface extract of *P. gingivalis*, the soluble component of the surface fraction, and the insoluble component of the surface fraction.

A Western immunoblot with αrGDH demonstrated that GDH was present in the surface extract and in both the soluble and insoluble surface fractions derived from the surface extract. NAD-dependent GDH activity was measured spectrophotometrically, and the activity in each fraction was standardized to 100 ml of broth culture. GDH activity of 467 U was found with intact whole cells; the activity was reduced to 147 U in cells which had been treated to remove loosely associated membrane material. The surface extract contained 115 U of GDH activity. When the surface extract preparation was fractionated, 85 U was found in the soluble fraction and 7 U was found in the insoluble fraction. The concomitant removal of the GDH protein and GDH activity from *P. gingivalis* by agitation of whole cells provides evidence that an

TABLE 1. Amino acid similarities between different GDHs

GDH source	Cofactor specificity[a]	Length[b]	% Amino acid identity[c]
Porphyromonas gingivalis ATCC 33277	NAD	445	100.0
Salmonella typhimurium	NADP	447	54.8
Escherichia coli	NADP	447	54.4
Giardia lamblia	NADP	449	52.4
Clostridium symbiosum	NAD	450	50.8
Saccharomyces cerevisiae	NADP	453	45.8
Clostridium difficile	NR	421	43.6
Peptostreptococcus asaccharolyticus	NR	421	24.0
Human origin	Dual	558	16.4
Chicken origin	Dual	503	15.7

[a] NAD, NAD-dependent GDH; NADP, NADP-dependent GDH; dual, dual cofactor specificity GDH; NR, not reported.
[b] Total number of amino acids making up the monomeric subunit protein.
[c] Number of identical amino acids, expressed as a percentage of the total number of residues in the *P. gingivalis* GDH.

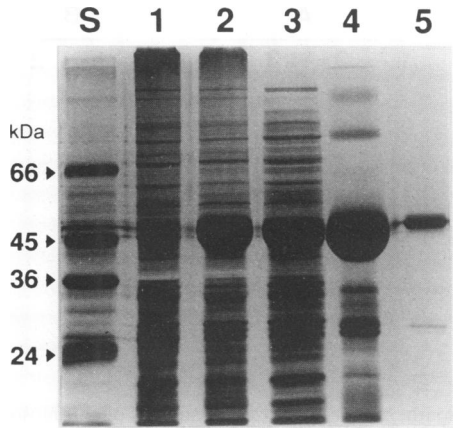

FIG. 3. Purification of the recombinant protein rGDH. An SDS–10% polyacrylamide gel stained with silver nitrate is shown. Lanes: S, protein standards; 1, whole-cell lysate of *E. coli* JM83/pUC18; 2, whole-cell lysate of *E. coli* BA3; 3, soluble fraction of BA3; 4, post-MonoQ anion-exchange chromatography; 5, post-Superose 12 gel filtration chromatography. Numbers on the left indicate molecular mass in kilodaltons.

active form of GDH enzyme is associated with the outer membrane.

Insertional inactivation of *gdh*. A deletion-replacement disruption was chosen as the means to alter the *gdh* structural gene (Fig. 5). A 400-bp *Acc*I-*Acc*I fragment was removed from the center portion of the *gdh* gene (Fig. 2), and a 3.4-kb fragment containing the *Bacteroides* Em^r gene was inserted. To facilitate transfer of the deletion-disrupted *gdh* gene into *P. gingivalis*, the suicide shuttle vector pJOE1 was constructed for DNA mobilization from *E. coli* to *P. gingivalis*.

Plasmid pJOE1 carried an *E. coli* origin of replication and a kanamycin resistance (Km^r) gene for maintenance and selection in *E. coli*. The Km^r marker is not functional in *Bacteroides* species. The *Bacteroides* Em^r gene confers Em^r on *Bacteroides* species but is not active in *E. coli*. Plasmid pJOE1 carried DNA elements for mobilization of the plasmid by transfer factors expressed from the helper plasmid R751. These transfer functions originated from *Bacteroides* conjugal elements; however, they do appear to mediate DNA transfer into *P. gingivalis* as demonstrated by the mobilization of shuttle vector pNJR5 (used in the construction of pJOE1) from *E. coli* to *P. gingivalis* by Maley et al. (26). In the absence of a *Bacteroides* origin of

TABLE 2. GDH activity of rGDH[a]

Sample	NAD-dependent GDH activity (U[b]/mg of protein)	NADP-dependent GDH activity (U[b]/mg of protein)
E. coli JM83/pUC18 soluble fraction[c]	0.03	0.35
E. coli BA3 soluble fraction[c]	946	0.60
Purified rGDH	1,139	0.62
Purified bovine GDH	52	13

[a] As determined by a spectrophotometric assay with α-ketoglutarate as substrate and NAD(P)H as cofactor.

[b] One unit will reduce 1.0 μmol of α-ketoglutarate to L-glutamate per min at 25°C and pH 8.1 in the presence of ammonium ions and NAD(P)H.

[c] Supernatant fraction after ultracentrifugation (100,000 × *g*, 1 h, 4°C) of disrupted cells.

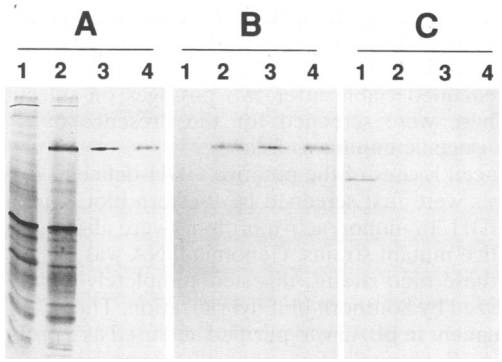

FIG. 4. Analysis of rGDH and native GDH by nondenaturing PAGE and GDH zymography. (A) Polyacrylamide (7.5%) gel stained with silver nitrate. (B) Zymogram showing NAD-dependent GDH activity. (C) Zymogram showing NADP-dependent GDH activity. L-Glutamate was used as substrate for detection of GDH activity. Lanes: 1, *E. coli* JM83/pUC18 soluble fraction; 2, *E. coli* BA3 soluble fraction; 3, purified rGDH; 4, partially purified native GDH.

replication, pJOE1 was not expected to replicate in *P. gingivalis*.

The generation of isogenic mutants of *P. gingivalis* was achieved by transferring the suicide shuttle vector pJOE1 into the parent organism by conjugal mating of *E. coli* XL1 donor cells carrying the plasmids R751 and pJOE1 with *P. gingivalis* ATCC 33277. Transconjugants were selected on prereduced BHI-blood agar supplemented with erythromycin and gentamicin.

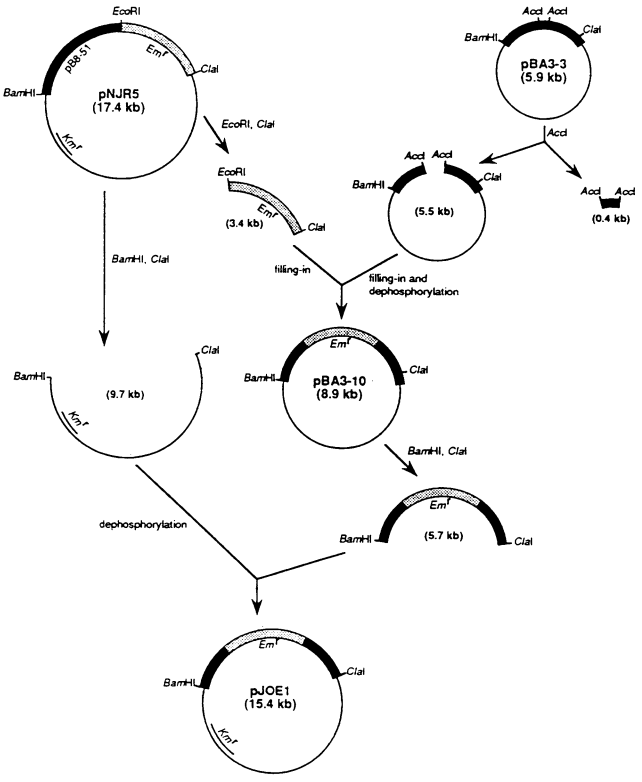

FIG. 5. Construction of the suicide shuttle vector pJOE1 for inactivation of the *P. gingivalis gdh* gene (see text for further details).

The *E. coli* XL1/R751,pJOE1-*P. gingivalis* ATCC 33277 mating yielded a total of 102 Em^r *P. gingivalis* colonies on the primary selection plates. Of these colonies, 53 *P. gingivalis* strains remained viable after two passages on selective medium. These were screened for the presence of a GDH-deficient isogenic mutant as follows.

Whole-cell lysates of the putative GDH-deficient *P. gingivalis* mutants were first screened by Western blot immunoassay with αrGDH. Immunoreactive proteins were absent in nine of the putative mutant strains. Genomic DNA was isolated from each of these nine strains, digested completely with *Hin*dIII, and analyzed by Southern blot hybridization. The 3.7-kb insert DNA fragment in pBA3 was purified and used as a probe. Only one strain, *P. gingivalis* E51, showed a hybridization pattern consistent with complete loss of the normal *gdh* gene and insertion of the modified *gdh* gene into the bacterial chromosome. The results of this analysis of *P. gingivalis* E51 are discussed below.

Genetic characterization of *P. gingivalis* E51. The nature of the DNA rearrangement which had occurred in the chromosome as a result of homologous recombination between pJOE1 and chromosomal DNA was investigated. A double crossover event would result in the exchange of an intact *gdh* gene for the modified gene carried on the suicide vector. A single crossover event would result in gene duplication. *P. gingivalis* E51 genomic DNA was isolated and analyzed by Southern blot hybridization.

Genomic DNA was completely digested with either *Hin*dIII or *Pst*I and then was probed with the 3.7-kb *Pst*I-*Pst*I insert DNA fragment (probe 1) isolated from pBA3. If a single crossover event had occurred between the *P. gingivalis* chromosome and pJOE1 (resulting in gene duplication), the probe was expected to hybridize with fragments of >15 and >3.4 kb in the *Hin*dIII-digested genomic DNA from *P. gingivalis* E51 and 13.8 and 3.2 kb in the *Pst*I-digested genomic DNA from *P. gingivalis* E51. However, if a double crossover event had occurred, hybridizing bands of >3.4 and >2.6 in *Hin*dIII-digested genomic DNA from *P. gingivalis* E51 and 6.7 kb in *Pst*I-digested genomic DNA from *P. gingivalis* E51 were expected. The *P. gingivalis* E51/*Hin*dIII sample exhibited two hybridizing bands at 6.5 and 5.6 kb (Fig. 6A, lane 2). A single band at 7.1 kb was observed in the *P. gingivalis* E51/*Pst*I sample (Fig. 6A, lane 4). These observations were consistent with the results expected if allelic exchange had occurred because of a double-crossover event between pJOE1 and *P. gingivalis* chromosomal DNA.

P. gingivalis E51 genomic DNA digested with *Pst*I was also probed with a 3.4-kb *Eco*RI-*Cla*I fragment (probe 2), isolated from pNJR5, containing the *Bacteroides* Em^r gene. A single hybridizing band of 7.1 kb was observed in the *P. gingivalis* E51/*Pst*I sample (Fig. 6B, lane 2). DNA sequences homologous to the probe were not detected in the genomic DNA sample from *P. gingivalis* ATCC 33277 (Fig. 6B, lane 1).

The results from Southern hybridization analysis of genomic DNA demonstrated that in *P. gingivalis* E51 the intact chromosomal *gdh* gene had been exchanged for the deletion-disrupted allele. *P. gingivalis* E51 therefore represented a true isogenic mutant with an altered gene for the cell surface-associated GDH subunit protein.

Characterization of *P. gingivalis* E51. Whole cells of wild-type and mutant *P. gingivalis* were analyzed by nondenaturing gel electrophoresis. A comparison of the protein profiles revealed that a prominent protein in the wild-type strain was absent from the mutant strain (Fig. 7A). This protein exhibited NAD-dependent GDH activity (Fig. 7B) and was reactive with antibodies to rGDH as shown in a Western immunoblot (Fig. 7C). NAD-dependent GDH activity was not detected in the *P. gingivalis* E51 sample, nor were there proteins reactive with αrGDH. It appeared that *P. gingivalis* E51 did not express a detectable intact or truncated GDH molecule. Immunogold bead labelling of whole cells with αrGDH verified that GDH is present on the cell surface of wild-type *P. gingivalis* and is missing in the mutant *P. gingivalis* E51 (Fig. 8).

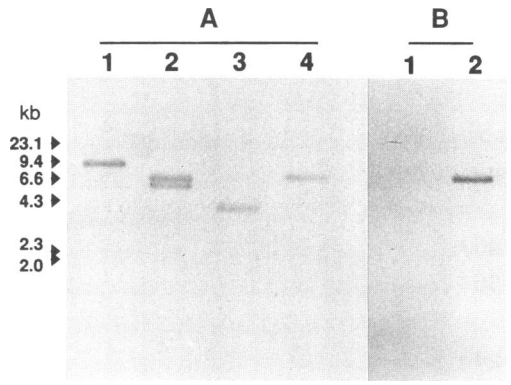

FIG. 6. Southern blot hybridization analysis of chromosomal DNA from *P. gingivalis* strains ATCC 33277 and E51. Two different DNA fragments were used as probes. (A) The 3.7-kb *gdh*-containing *Pst*I-*Pst*I fragment of pBA3 used as probe. Lanes: 1, *Hin*dIII-digested genomic DNA from *P. gingivalis* 33277; 2, *Hin*dIII-digested genomic DNA from *P. gingivalis* E51; 3, *Pst*I-digested genomic DNA from *P. gingivalis* 33277; 4, *Pst*I-digested genomic DNA from *P. gingivalis* E51. (B) The 3.4-kb *Eco*RI-*Cla*I fragment of pNJR5 containing the *Bacteroides* Em^r gene used as probe. Lanes: 1, *Pst*I-digested genomic DNA from *P. gingivalis* 33277; 2, *Pst*I-digested genomic DNA from *P. gingivalis* E51. Numbers on the left indicate size in kilobases.

GDH activity of the wild-type and mutant *P. gingivalis* strains was measured spectrophotometrically with α-ketoglutarate and NADH. A fraction containing soluble proteins from the wild-type strain exhibited GDH activity of 70 U/mg of protein. In contrast, a sample containing soluble proteins from the mutant strain showed almost negligible GDH activity (0.05

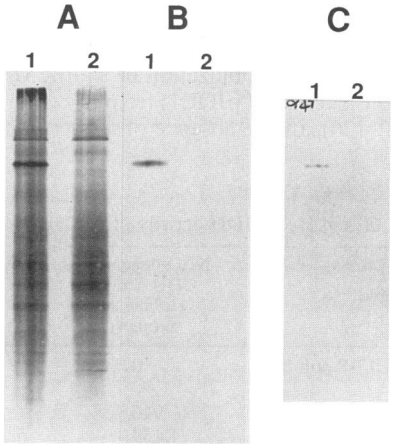

FIG. 7. Analysis of nondenatured samples of *P. gingivalis* ATCC 33277 and E51. (A) Polyacrylamide (7.5%) gel stained with silver nitrate. (B) Zymogram showing NAD-dependent GDH activity. (C) Western blot of the gel reacted with αrGDH. L-Glutamate was used a substrate for detection of GDH activity. Lanes: 1, *P. gingivalis* 33277; 2, *P. gingivalis* E51.

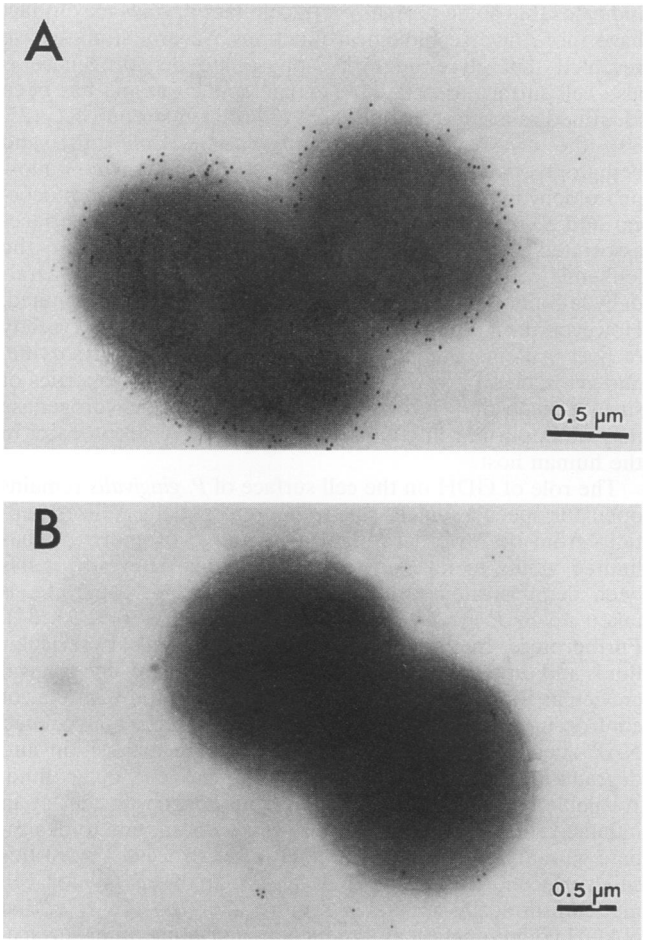

FIG. 8. Immunogold bead labelling of GDH on the surface of *P. gingivalis* ATCC 33277 (A) and E51 (B). *P. gingivalis* cells were incubated with αrGDH followed by gold beads conjugated to goat anti-rabbit immunoglobulin G. The cells were negatively stained with ammonium molybdate and viewed by electron microscopy.

U/mg). These measurements support the results shown in the GDH zymogram, in which NAD-dependent GDH activity was seen in the wild-type *P. gingivalis* sample but not in the mutant (Fig. 7B).

Because GDH can be involved in catabolic metabolism, it was of interest to examine whether loss of NAD-dependent GDH activity resulted in a change in metabolic end products. *P. gingivalis* typically produces propionic and butyric acids, and some strains generate succinic acid (9). Culture supernatants of wild-type and mutant strains were analyzed for volatile and nonvolatile fatty acids by gas chromatography. Both strains showed similar levels of propionic, butyric, and succinic acids.

Both parent and mutant strains were equivalent in their ability to aggregate erythrocytes and to coaggregate with *Actinomyces naeslundii* and *Treponema denticola.*

The loss of GDH has a marked impact on the growth of the organism. When grown in BHI broth, *P. gingivalis* E51 had a significantly longer lag period and a generation time of 4.3 h compared with 2.7 h for the parent and reached a maximum absorbance of 1.0, whereas the parent entered the stationary growth phase at an absorbance of 1.5. Addition of erythromycin had no effect on the growth rate of *P. gingivalis* E51.

DISCUSSION

The structural gene for the GDH protein of *P. gingivalis*, along with a promoter sequence for transcriptional initiation, was localized to a 2.0-kb *Cla*I-*Bgl*II portion of the original 3.7-kb cloned DNA fragment (Fig. 1). The nucleotide sequence of the 2.0-kb *Cla*I-*Bgl*II region was determined (Fig. 2). Potential −35 and −10 regions as promoter elements for the *gdh* gene and a prokaryotic rbs were identified.

The *gdh* gene was found to consist of a 1,338-bp ORF which encodes a protein of 445 amino acids (Fig. 2). From the deduced amino acid sequence, the molecular mass of the translated protein was calculated to be 49,243 Da. Previous analysis of the rGDH and native GDH proteins demonstrated two forms of GDH with apparent molecular masses of 48 and 51 kDa (22). It appeared that the 48-kDa form resulted from degradation of the higher-molecular-mass molecule, although posttranslational modification of the 48-kDa protein to generate the 51-kDa species could not be ruled out. It was hoped that nucleotide sequencing would assist in determining the size of the protein expressed from the *gdh* gene. However, given the relative inaccuracy of molecular mass estimation by SDS-PAGE, it is not clear whether the deduced 49-kDa protein corresponded to the 48-kDa species or the 51-kDa species observed in GDH protein preparations.

The deduced amino acid sequence for GDH did not reveal the presence of a leader sequence typical of proteins transported through the membrane. However, several exported proteins from other organisms also appear to lack a recognizable NH_2-terminal signal peptide. These include flagellin (29) and a surface-layer protein (16) from *Caulobacter crescentus*, surface-layer proteins of *Rickettsia prowazekii* (5) and *Campylobacter fetus* (3), hemolysin of uropathogenic *E. coli* strains (4), metalloprotease B of *Erwinia chrysanthemi* (10, 11), and the surface-located glyceraldehyde-3-phosphate dehydrogenase of group A streptococci (25, 30). A hydrophobic region of approximately 20 residues at the COOH terminus of the GDH protein may play a role in localizing the molecule to the *P. gingivalis* cell surface (i) by acting as an anchor inserted into the lipid bilayer of the outer membrane or (ii) by hydrophobic interaction with hydrophobic regions on the outer surface of *P. gingivalis.*

A data base search for proteins homologous to GDH revealed significant sequence similarity to the subunit protein of GDH enzymes isolated from a variety of sources (Table 1). It appeared that the GDH protein was the monomeric subunit of a *P. gingivalis* GDH enzyme. Curtis and coworkers purified the native GDH molecule (42) and determined by gel filtration chromatography that the molecular mass was approximately 300,000 Da (7a). This is consistent with the reported molecular masses of other hexameric GDH enzymes (12, 17). With few exceptions, the subunit protein of the hexameric enzyme is in the range of 45 to 50 kDa. It would be consistent with observations of other GDH enzymes that six GDH monomers associate to form the active oligomeric *P. gingivalis* NAD-dependent GDH enzyme.

Purified rGDH was analyzed for GDH activity with α-ketoglutarate as substrate. When NADH was supplied as the cofactor, the recombinant protein exhibited a high level of dependent GDH activity (Table 2). In contrast, a significantly lower level of GDH activity was detected in the presence of NADPH as the cofactor. Use of the same assay procedure to measure the activity of NAD-dependent GDHs of several *Clostridium botulinum* strains revealed that proteolytic strains had specific activities significantly higher than those of nonproteolytic strains (20). The vigorous activity of NAD-depen-

dent GDHs in bacteria such as *P. gingivalis* and proteolytic *C. botulinum* suggests that these enzymes are important in anaerobes which are dependent on fermentation of amino acids for energy.

In terms of the physiological role of GDHs, the cofactor specificity of a particular GDH generally dictates the type of metabolic pathway in which the enzyme is involved. NADP-dependent GDHs usually play an anabolic role in which they catalyze the assimilation of ammonia by reductive amination of α-ketoglutarate to form L-glutamate. This amino acid can then be used to synthesize other amino acids by transamination. NAD-dependent GDHs normally serve a catabolic function, catalyzing the oxidative deamination of L-glutamate to α-ketoglutarate, which is then incorporated into the energy-generating system of the organism. The dependency on NAD for GDH activity suggests that the *P. gingivalis* GDH is involved in degradation rather than biosynthesis of glutamate. This would be in keeping with the dependence of the organism on fermentation of amino acids as its principal energy source.

When NADP was supplied as a cofactor, the NADP-dependent GDH of *E. coli* (17) was detected in the JM83/pUC18 sample (Fig. 4C) but not in the *E. coli* BA3 preparation. The absence of *E. coli* NADP-dependent GDH for generation of glutamate in clone BA3 may be due to the presence of a high level of active recombinant GDH. As mentioned above, NAD-dependent GDHs generally function in a catabolic role; however, it has been shown that under certain conditions in vivo they will catalyze the reverse reaction, thereby playing a role in glutamate biosynthesis. A mutant strain of *Saccharomyces cerevisiae* lacking both NADP-dependent GDH and glutamate synthase (the enzymes involved in the two pathways for glutamate biosynthesis) was a glutamate auxotroph. However, when a *S. cerevisiae* gene encoding a NAD-dependent GDH was introduced into the mutant strain on a high-copy-number plasmid, a high level of recombinant NAD-dependent GDH was expressed and a high level of glutamate was synthesized (27). In a similar study conducted by Snedecor et al. (38), a NAD-dependent GDH cloned from *Peptostreptococcus asaccharolyticus* was able to substitute for the missing biosynthetic NADP-dependent GDH in *E. coli gdh* mutants. A very active *P. gingivalis* rGDH is highly expressed in *E. coli* BA3. It is possible that BA3 utilizes the NAD-dependent recombinant enzyme for biosynthesis of glutamate in the cell, thereby dismissing the need for the *E. coli* NADP-dependent GDH.

A study of *P. gingivalis* E51 showed that loss of GDH slowed the growth of the organism and resulted in lower cell yields in cultures growing in BHI broth. Given that the *P. gingivalis* GDH is a NAD-dependent enzyme and that glutamate is an important substrate in energy metabolism, it is likely that the GDH-negative mutant is unable to utilize the available glutamate. This suggests an important role for glutamate in energy metabolism and implies that other energy-yielding amino acids are not present in adequate supply to compensate for the loss in ability to ferment glutamate.

A review of the literature reveals that bacterial GDH enzymes are located in the cytoplasm or the cytoplasmic membrane of the cell. The NAD-dependent GDH of *Pseudomonas aeruginosa* is associated with the cytoplasmic membrane (21), and NAD-specific GDHs of eukaryotic organisms are located in mitochondria (17). It is therefore not unreasonable that the NAD-dependent *P. gingivalis* GDH could be membrane associated; however, it is unusual that at least some of the enzyme is on the outside of the cell and that the externalized enzyme exists in an active form.

Our report joins an increasing body of evidence which indicates that some normally intracellular enzymes may in fact have more diverse biological functions. Several studies have revealed that glyceraldehyde-3-phosphate dehydrogenase is also cell surface associated. This metabolic enzyme has been identified as a surface component of group A streptococci (25, 30), the invasive parasite *Schistosoma mansoni* (18), and hematopoietic cells (1). Furthermore, glyceraldehyde-3-phosphate dehydrogenase activity was detected on the streptococcal and *S. mansoni* cell surface, indicating that the surface-associated form is enzymatically active. No role for the leukemic cell or schistosomal membrane-associated glyceraldehyde-3-phosphate dehydrogenase has been determined. However, the streptococcal enzyme appears to bind to a variety of host proteins, namely, plasmin (25), fibronectin, lysozyme, and cytoskeletal proteins (30). These adherence properties of surface-associated glyceraldehyde-3-phosphate dehydrogenase may be important in colonization of group A streptococci in the human host.

The role of GDH on the cell surface of *P. gingivalis* remains open for speculation. *P. gingivalis* preferentially utilizes peptides from the external milieu as a source of energy. It has limited ability to take up free amino acids. However, it has been demonstrated that glutamate, as well as aspartate, is taken up by *P. gingivalis* as readily as peptides (15, 33, 34). Furthermore, free glutamate is present in gingival crevicular fluid and appears to be significantly elevated in gingival crevicular fluid of periodontal patients compared with that of control individuals (39). A *P. gingivalis* surface-associated NAD-specific GDH may play a role in the acquisition and degradation of L-glutamate from gingival crevicular fluid. Available NAD, possibly liberated from phagocytic cells at an inflamed site, would enable the *P. gingivalis* enzyme to deaminate glutamate, generating NADH in the process. Use of this cell surface-located NADH as a source of energy for cell metabolism seems unlikely. A more likely possibility is that NADH could function as a reductant, activating other surface-associated components. Some outer membrane-associated proteases of *P. gingivalis* require a reducing environment for activity (19). The activity of these proteases appears to be dependent on free sulfhydryl groups and is significantly enhanced in the presence of reducing agents. The hemagglutinating activity of *P. gingivalis* is elevated by reducing agents (37). Another potential role for the surface-located GDH may be as an adhesin for binding to immobilized host and bacterial proteins through the glutamate-binding domain.

Maley et al. (26) reported difficulties in maintaining *P. gingivalis* containing pNJR5 (the plasmid confers Em^r on *P. gingivalis*) on erythromycin-containing medium. They postulated that this was due to poor expression of the plasmid-borne Em^r selection marker in *P. gingivalis*. The gene for erythromycin selection present in the chromosome of *P. gingivalis* E51 originated from pNJR5; however, growth of E51 was the same whether or not erythromycin was included in the medium. In addition, with the exception of strain E51, the other Em^r *P. gingivalis* mutants obtained in this study all grew in the presence of erythromycin at a rate similar to that of the wild type growing in a medium without erythromycin. Expression of the *Bacteroides* Em^r gene therefore did not appear to hinder the growth of *P. gingivalis* strains in which the antibiotic marker was present in the bacterial chromosome. It is possible that in our *P. gingivalis* mutants, expression of the antibiotic resistance marker was from the *P. gingivalis* promoter for the *gdh* gene. It may also be possible that *P. gingivalis* strains carrying the plasmid pNJR5 are difficult to maintain because of the instability of the plasmid itself rather than poor expression of the Em^r gene.

Further examination of the surface-associated GDH protein and characterization of the GDH-deficient *P. gingivalis* mutant are clearly necessary to elucidate the role of this novel cell surface component.

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