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The *gnd* Gene Encoding a Novel 6-Phosphogluconate Dehydrogenase and Its Adjacent Region of *Actinobacillus actinomycetemcomitans* Chromosomal DNA

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A 10-kb DNA fragment containing the *gnd* gene from *Actinobacillus actinomy-cetemcomitans* Y4 was isolated and sequenced. The structural *gnd* gene codes for 6-phosphogluconate dehydrogenase that consists of 484 amino acids. In contrast to the *gnd* gene in *Escherichia coli, Salmonella typhimurium,* or *Klebsiella pneumoniae,* the *gnd* gene of *A. actinomycetemcomitans* was not located in the *rfb* or *cps* operon. The *zwf* gene encoding glucose 6-phosphate dehydrogenase, which is another enzyme consisting of pentose-phosphate pathway, sided at 3.8-kb upstream from the *gnd* gene. A phylogenetic tree based on sequence analyses showed higher homology of 6-phospho-gluconate dehydrogenase of *A. actinomycetemcomitans* with the eucaryotic enzymes rather than with bacterial enzymes.

6-Phosphogluconate dehydrogenase (6-phospho-D-gluconate: NADP oxido-reductase [decarboxylating], EC 1. 1. 1. 44; 6PGDH) and glucose 6-phosphate dehydrogenase (Glucose-6-phosphate: NADP oxdoreductase, EC 1. 1. 1. 49; G6PD) are enzymes in the pentose phosphate pathway (1). Its primary functions are the synthesis of ribulose 5-phosphate for biosynthesis of nucleotides, aromatic amino acids, vitamins, and cell wall constituents, and production of NADPH for reductive biosynthesis. The *gnd* genes were cloned and sequenced from several bacteria. The 6PGDH amino acid sequences are highly conserved among those bacteria, with 56-96% sequence identity.

Actinobacillus actinomycetemcomitans is a facultative gram-negative rod, and is considered to be associated with localized juvenile periodontitis (2) and adult periodontitis (3). Several oral bacteria, e. g., *Streptococcus mutans* (4), *Porphyromonas gigivalis* (5), and *Prevotella intermedia* (5) do not exhibit 6PGDH and G6PD, although 6PGDH plays an important role as a glucose and gluconate catabolic enzyme in many microorganisms. Recently, Sweeney et al. (6) reported that the utilization of gluconate is an important element in colonization by *Escherichia coli* of streptomycin-treated mouse large intestine. Little is known about glucose metabolism of *A. actinomycetemcomitans* to date, despite importance of 6PGDH and G6PD in carbon metabolism.

To analyze the *gnd* gene and its flanking region, the *gnd* gene was cloned from *A. actinomycetemcomitans* Y4 and its nucleotide sequence was determined.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* DH5 was used as a host strain for cosmid library. *E. coli* XL1-Blue was used for subcloning of fragments. *E. coli* RW231 [*trpR kdgR lacZ*(Am) *trpA9605* Δ(*edd-zwf*)22 Δ(*sbcB-his-gnd-rfb*) *recA rpsL20*] (7) (Fig. 1) was used for detection of 6PGDH activity in nondenaturing polyacrylamide gels. *E. coli* strains were grown in Luria-Bertani medium at 37°C. Media were supplemented with antibiotics as required.

DNA isolation, polymerase chain reaction (PCR), and sequence. Chromosomal DNA was extracted and purified from *A. actinomycetemcomitans* Y4 as described previously (8). PCR was carried out on approximately 30 ng of chromosomal DNA. To amplify a *gnd* fragment of *A. actinomycetemcomitans* Y4 for gene cloning, degenerate oligonucleotides were synthesized according to the amino acid sequences conserved in reported 6PGDHs: 5′-GARTAYGGNGAYATGCA-3′ (5′ primer) and 5′-TARTCRCGYTGNGCYTG-3′ (3′ primer). The amplified fragment was cloned into pGEM-T (Promega) and sequenced. The nucleotide sequence was determined by the dideoxy chain termination method using the Taq dye primer cycle sequencing kit and ABI 373A DNA sequencer (Perkin-Elmer Cetus).

Cosmid library and colony hybridization. Two cosmid gene banks were constructed by using chromosomal DNA from *A. actinomycetemcomitans* Y4. The chromosomal DNA was partially digested with *Eco*RI or *Bam*HI, and fragments of 35 to 45-kb were cloned into the *Eco*RI or *Bam*HI site in pMBLcos (9), respectively. The cloned fragment amplified by PCR was labeled by random priming with

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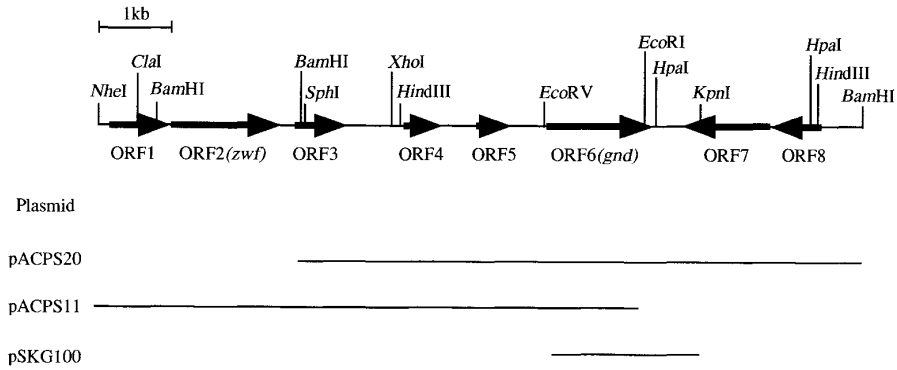

FIG. 1. Physical map of the cloned insert and complementation of *E. coli* RW231. Arrows indicate direction of transcription.

digoxigenin-11-dUTP (Boehringer Mannheim) and used as a probe to screen the genomic library.

Detection of enzymatic activity. 6PGDH activity in nondenaturing polyacrylamide gel was detected as described previously (10).

Nucleotide sequence accession number. The sequence data reported in this paper have been submitted to the DDBJ under the accession No. D88189.

RESULTS AND DISCUSSION

To obtain a probe for library screening, we performed a genomic PCR using a pair of the *gnd* primers and *A. actinomycetemcomitans* Y4 genomic DNA template. Two highly conserved amino acid stretches were selected by comparison of previously reported sequences of the *gnd* genes. They were back-translated into nucleotide sequence on the basis of the codon usage of the *A. actinomycetemcomitans* Y4 *groESL* operon (8). The PCR produced a single band of 770 bp. The sequencing of the fragment revealed that it contained a continuous ORF, which showed 56.3% sequence identity with *E. coli* 6PGDH. Four independent genomic clones bearing the *gnd* gene were isolated from 800 colonies of the libraries. Of those, two clones contained the same 10-kb *Bam*HI fragment and the rest contained the same 15-kb *Eco*RI fragment. The former two plasmids were designated pACPS20 and the latter plasmids were designated pACPS11.

The 6.0-kb *Hin*dIII fragment which hybridized with the probe was subcloned into pMCL200 (9) and the complete sequence of both strands of the insert was determined. The coding region corresponding to the *A. actinomycetemcomitans gnd* gene is shown in Fig. 2. Several inverted repeats were found in the upstream region from the initiation codon. The function of these inverted repeats in the promoter region of the *gnd* gene is unknown. In *E. coli* and *Salmonella enterica,* the segment of *gnd* mRNA between codons 67 and 78 is complementary to an extensive portion of the *gnd* ribosome-binding site, and this region plays an important role in growth-rate-dependent regulation of expression of 6PGDH as a cis-acting antisense RNA (11). Such a structure was not located in the *A. actinomycetemcomitans gnd* gene.

A comparison of the derived amino acid sequence of the 6PGDH polypeptide of *A. actinomycetemcomitans* with the sequences available in the data bank revealed several conserved regions (Fig. 3). One of the regions (amino acid residues 126 to 136) contains the highly conserved G-X-G-X-X-G fingerprint pattern for NADP(H) or NAD(H) binding (12). The amino acid sequence shows 52, 53, 52, and 52% identity with that of *E. coli, Shigella flexneri, S. enterica,* and *Klebsiella pneumoniae* 6PGDHs, respectively, whereas among this taxonomic group of *Enterobacteriaceae* 6PGDHs shows over 93% identities. Figure 4 presents phylogenetic tree for procaryotic and eucaryotic 6PGDHs previously reported. Interestingly, 6PGDH of *A. actinomycetemcomitans* showed higher homology with the eucaryotic enzymes rather than with bacterial enzymes. The origin of this peculiar structure is unknown.

This result raised the possibility that the gene product is a 6PGDH homologue but not exhibit 6PGDH activity. Plasmid-encoded 6PGDH activity was determined by complementation of the defect in *E. coli.* The cells harboring pSKG100, a pBluescript SK derivative plasmid containing *A. actinomycetemcomitans gnd,* produced a significant amount of 47.0 kDa protein which was not observed in the cells harboring the vector (Fig. 5A). This apparent molecular weight was in good agreement with the predicted molecular weight from the deduced amino acid sequence of *A. actinomycetemcomitans* 6PGDH. Only the transformants harboring the plasmids containing the *gnd* gene exhibited $6PGDH^+$ phenotype (Fig. 5B).

The *gnd* gene was shown to map next the *rfb* gene cluster, which is responsible for the synthesis of lipopolysaccharide antigen in *E. coli* (13), *S. flexneri* (14) and *S. enterica* serover *typhimurium* (15), or the *cps* gene cluster, which is responsible for the synthesis of capsular polysaccharide in *K. pneumoniae* (16). *A. actinomycetemcomitans* also produces capsular-like

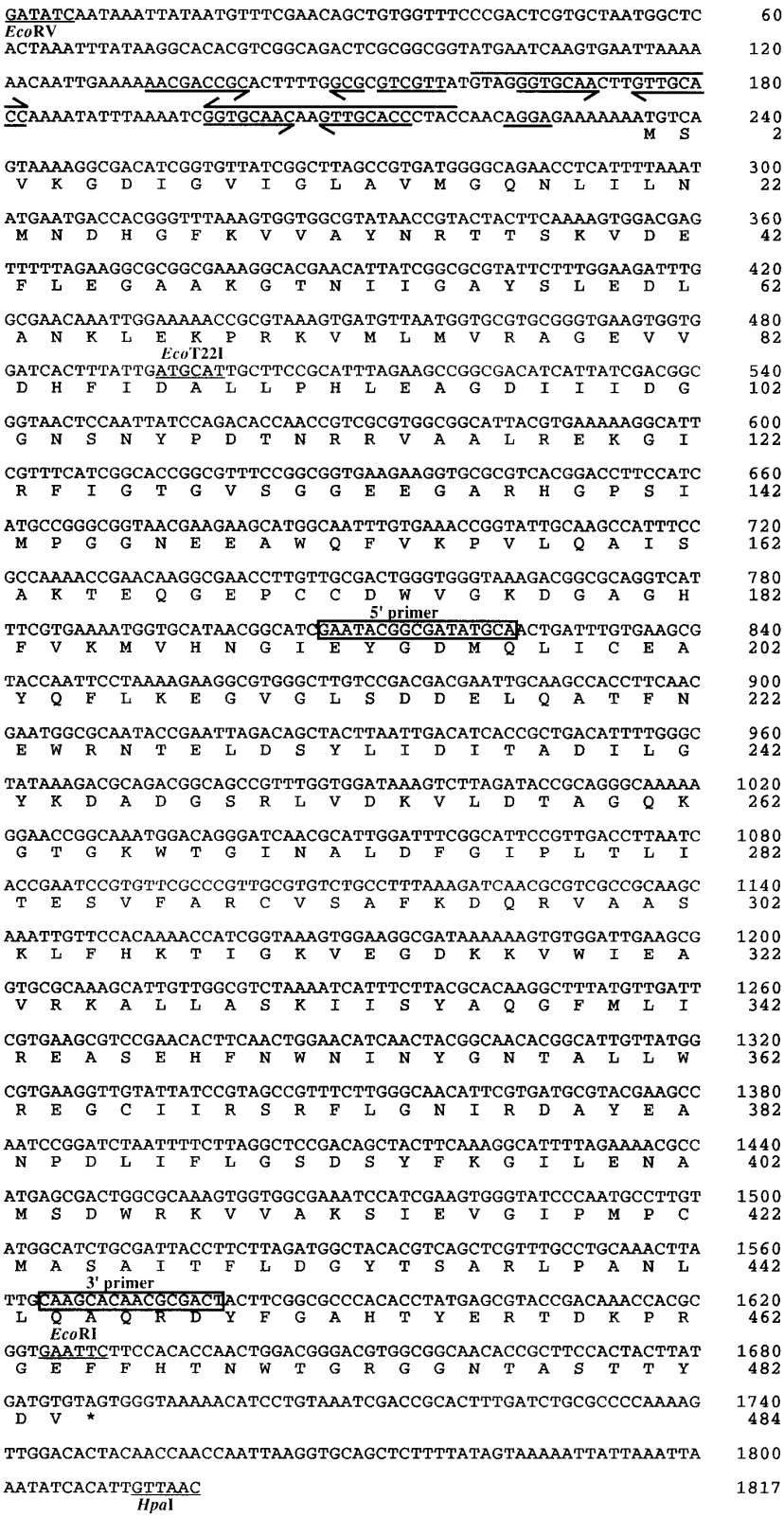

FIG. 2. The nucleotide sequence of the *A. actinomycetemcomitans gnd* gene, its flanking regions and deduced amino acid sequence. The putative ribosome binding site before the ATG start codon is indicated by an underline. The inverted repeats found in the upstream sequence are indicated by arrows.

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Aac  MSVKGDIGVIGLAVMGQNLILNMNDHGFKVVAYNRTTSKVDEFLEGAAKG
Kpn  **-*QQ***V*M****R**A**IESR*YT*SVF**SRE*TE*VI-AENT*
Eco  **-*QQ***V*M****R**A**IESR*YT*SIF**SRE*TE*VI-AENP*
Sen  **-*QQ***V*M****R**A**IESR*YT*SVF**SRE*TE*VI-AENP*
Sfl  **-*QQ***V*M****R**A**IESR*YT*SIF**SRE*TE*VI-AENP*
Scy  *AL-QQF*LI******E**A**IERN**SLTV****AE*TEA*MADRAQ*

Aac  TNIIGAYSLEDLANKLEKPRKVMLMVRAGEVVDHFIDALLPHLEAGDIII
Kpn  KKLVPY*TVQEFVES**T**RIL***K**AGT*SA**S*K*Y*DK*****
Eco  KKLVPY*TVKEFVES**T**RIL***K**AGT*AA**S*K*Y*DK*****
Sen  KKLVPY*TVKEFVES**T**RIL***K**AGT*AA**S*K*Y**K*****
Sfl  KKLAPY*TVKEFVES**T**RIL***K**AGT*AA**S*K*Y*DK*****
Scy  K**VP******FVAS**R**RILV**K**GP**AVVEQ*K*L*DP**L**

Aac  DGGNSNYPDTNRRVAALREKGIRFIGTGVSGGEEGARHGPSIMPGGNEEA
Kpn  ****TFFQ**I**NRE*SAE*FN**************LK********QK**
Eco  ****TFFQ**I**NRE*SAE*FN**************LK********QK**
Sen  ****TFFQ**I**NRE*SAE*FN**************LK********QKD*
Sfl  ****TFFQ**I**NRE*SAE*FN**************LK********QK**
Scy  *****LFT**E***KD*EAL*LG*M*M*********LN***L****TQA*

Aac  WQFVKPVLQAISAKTEQGEPCCDWVGKDGAGHFVKMVHNGIEYGDMQLIC
Kpn  YEL*A*I*KQ*A*VA*D****VTYI*A*****Y****************A
Eco  YEL*A*I*TK*A*VA*D****VTYI*A*****Y****************A
Sen  YEL*A*I*TK*A*VA*D****VTYI*A*****Y****************A
Sfl  YEL*A*I*TK*A*VA*D****VTYI*A*****Y****************A
Scy  YEA*SRSVPT*A*QVDD*-**VTYI*PG*S**Y****************A

Aac  EAYQFLKEGVGLSDDELQATFNEWRNT-ELDSYLIDITADILGYKDA--D
Kpn  ***AL**G*LA**NE**AQ**T**NEG-**S*******K**FTK**E--E
Eco  ***SL**G*LN*TNE**AQ**T**N*G-**S*******K**FTK**E--D
Sen  ***SL**G*LN**NE**AN**T**N*G-**S*******K**FTK**E--D
Sfl  ***SL**G*LN**NE**AQ**T**N*G-**S*******K**FTK**E--D
Scy  ***DL**SVA**NAS**HDV*AA*NKTP****F**E*****F*KV*DLGT

Aac  GSRLVDKVLDTAGQKGTGKWTGINALDFGIPLTLITESVFARCVSAFKDQ
Kpn  *KY***VI**E*AN*******SQSS**L*E**S*********YI*SL***
Eco  *NY***VI**E*AN*******SQS***L*E**S*********YI*SL***
Sen  *NY***VI**E*AN*******SQS***L*E**S*********YI*SL*AQ
Sfl  *NY***VI**E*AN*******SQS***L*E**S*********YI*SL***
Scy  *QP**ELI**A*******R**VET**EI*VAIPT*IAA*N**IL*SI*AE

Aac  RVAASKLFHKTIGKV-EGDKKVWIEAVRKALLASKIISYAQGFMLIREAS
Kpn  ******VLSGPQAQP-V***AGF**K**R**YLG**V******SQL*A**
Eco  ******VLSGRQAQP-A***AEF**K**R**YLG**V******SQL*A**
Sen  ******VLSGPKAQP-A***AEF**K**R**YLG**V******SQL*A**
Sfl  ******VLSGPQAQS-A***AEF**K**S**YLG**V******SQL*A**
Scy  *Q***EILSGP*TEPFS**RQAF*DS**D**YC***C*****MA*LAK**

Aac  EHFNWNINYGNTALLWREGCIIRSRFLGNIRDAYEANPDLIFLGSDSYFK
Kpn  DEY**DL***EI*KIF*A*****AQ**QK*T***AQ*AGIAN*LLAP***
Eco  *EY**DL***EI*KIF*A*****AQ**QK*T***AE**QIAN*LLAP***
Sen  DEYH*DL***EI*KIF*A*****AQ**QK*T***AE*A*IAN*LLAP***
Sfl  *EY**DL***EI*KIF*A*****AQ**QK*T***AE**QIAN*LLAP***
Scy  QVY*YGL*L*EL*RI*KG*****AG**NK*KQ**D*D*T*AN*LLAPE*R

Aac  GILENAMSDWRKVVAKSIEVGIPMPCMASAITFLDGYTSARLPANLLQAQ
Kpn  Q*ADDYQQAL*D***YAVQN***V*TVSA**AYY*S*R**V*****I***
Eco  Q*ADDYQQAL*D***YAVQN***V*TFSA*VAYY*S*RA*V*****I***
Sen  K*ADEYQQAL*D***YAVQN***V*TFSA*VAYY*S*RA*V*****I***
Sfl  Q*ADDYQQAL*D***YAVQN***V*TF*A*VAYY*S*RA*V*****I***
Scy  QTILDRQLA**R*I*IAA*R***V*AFSASLDYF*S*RASP-AQ**T***

Aac  RDYFGAHTYERTDKPRGEFFHTNWTGRGGNTASTTYDV
Kpn  *********K*****--EGV***E*LE
Eco  *********K*I**--EGV***E*LD
Sen  *********K*****--EGI***E*LE
Sfl  *********K*I**--EGV***E*LD
Scy  *TT-C****K--A*IALL**AMF
```

FIG. 3. Multiple alignment of 6PGDHs from *A. actinomycetemcomitans* (Aac), *K. pneumoniae* (Kpn), *E. coli* (Eco), *S. enterica* (Sen), *S. flexnieri* (Sf1), and *Synechococcus* sp. PCC7942 (Syn). The amino acid sequences of 6PGDHs were first progressively aligned using the program Clastal V 25. and further locally improved after visual inspection. The identities are indicated by asterisks (*) and conservative substitutions by plus symbols (+).

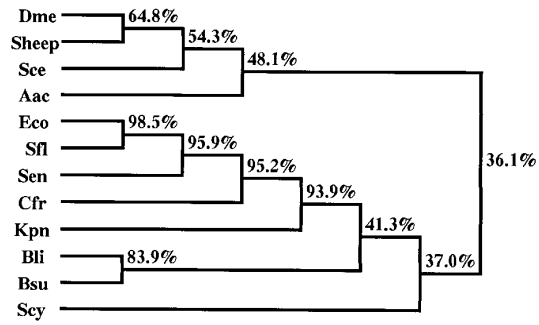

FIG. 4. Phylogenetic tree showing relationships of 6PGDHs. Distance between sequences was calculated by using the DNASIS package (Hitachi Software Engineering Co.). Abbreviations and accession numbers for these sequences are as follows: Dme, *Drosophila melanogaster* (M80598); Sheep, *Ovis orientalis aries* (999886); Sce, *Saccharomyces cerevisiae* (Z46631); Aac, *A. actinomycetemcomitans* Y4 (this study); Sfl, *S. flexneri* (U14468); Sen, *S. enterica* LT2 (M64332); Cfr; *Citrobacter freundii* (608061); Kpn, *K. pneumoniae* Chedid (D21242); Bli, *Bacillus licheniformis* (D31631), Bsu; *Bacillus subtilis* (D45242), Scy, *Synechococcus* PCC7942 (112845).

serotype-specific polysaccharide antigens consisting of 6-deoxyhexoses (17). The relatively frequent exchange of *gnd* within and among taxonomic groups of the *Enterobacteriaceae* is said to be result from its close linkage with genes that are subject to diversifying selection including those of the *rfb* region determining the structure of the polysaccharide (18). These cell surface polysaccharides of *A. actinomycetemcomitans* also play a key role in the resistance to phagocytosis and killing by human polymorphonuclear leukocytes (19). In the case of *A. actinomycetemcomitans*, however, no *rfb* or *cps* gene was located around the *gnd* gene (Fig. 1, Table 1). ORF2 is a homologue of the *zwf* gene encoding G6PD, and ORF3 is a *devB* homologue encoding G6PD isozyme. G6PD is a member of the oxidative branch of the pentose phosphate pathway which provides ribose for nucleotide biosynthesis and NADPH for reductive biosyntheses. It is not clear whether both the genes produce active G6PDs in *A. actinomycetemcomitans*. Two ORF7 and ORF8 located downstream from the *gnd*

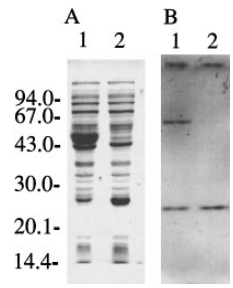

FIG. 5. Expression of the *A. actinomycetemcomitans gnd* gene in *gnd*-deficient *E. coli*. Coomassie blue-stained SDS-polyacrylamide gel (A) and G6PD activity-staining of a native polyacrylamide gel (B) of crude extracts of *E. coli* RW231 harboring pSKG100 (lane 1) and *E. coli* RW231 harboring the vector (lane 2). Total proteins of the cells (1 ml, OD_{600} = 0.1) were resolved on an SDS- (A) or native (B) 10.0% polyacrylamide gel. Positions of molecular mass markers are given in kilodaltons.

TABLE 1

Dedeuced Amino Acid Sequence Identities of Potential ORFs Found around *A. actinomycetemcomitans gnd* to Reported Homologues

Potential ORF identified around *A. actinomycetemcomitans gnd* gene	ORF1	ORF2	ORF3	ORF4	ORF5	ORF7	ORF8
Homologous gene	*cysQ*	*zwf*	*devB*	*lysR*	*vapD*	*fabF*	*FabG*
Bacterium	*E. coli*	*E. coli*	*Anabaena sp.*	*E. coli*	*Dichelobacter nodosus*	*E. coli*	*E. coli*
Protein sequence identity (%)	32.7	39.5	28.6	27.2	17.3	35.2	39.6
Protein function	Ammonium transport protein	G6PD	Putative G6PD isozyme	LysA activator protein	Unknown	β-Ketoacyl-ACP synthase IV	β-Ketoacyl-ACP reductase
Reference or accession no.	(20)	(11)	U14553	(21)	(22)	(23)	(24)

gene were homologues of *fabF* and *fabG*, respectively, which are involved in synthesis of fatty acid. Two ORFs located between *devB*-homologue and *gnd* showed a low degree of homology to *lysR* and *vapD*, and therefore functions of these genes are not distinct. ORF1 in the region upstream from the *zwf*-homologue showed 36% identity to the *cysQ* gene of *E. coli*.

Further functional analysis of the *A. actinomycetemcomitans gnd* gene and its gene product is currently in progress and should provide insight into the role of this unique 6PGDH in this organism.

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