Biochemical and Biophysical Research Communications **282,** 21–27 (2001)
doi:10.1006/bbrc.2001.4511, available online at http://www.idealibrary.com on IDEAL®

Cloning and Expression of Glucose 3-Dehydrogenase from *Halomonas* sp. α-15 in *Escherichia coli*

Katsuhiro Kojima, Wakako Tsugawa, and Koji Sode[1]
Department of Biotechnology, Tokyo University of Agriculture and Technology, 2-24-16 Naka-machi, Koganei, Tokyo 184-8588, Japan

Received February 1, 2001

The gene encoding glucose 3-dehydrogenase (G3DH) from *Halomonas* sp. α-15 was cloned and expressed in *Escherichia coli*. An open reading frame of 1686 nucleotides was shown to encode G3DH. The flavine adenine dinucleotide binding motif was found in the N-terminal region of G3DH. The deduced primary structure of G3DH showed about 30% identity to sorbitol dehydrogenase from *Gluconobacter oxydans* and 2-keto-D-gluconate dehydrogenases from *Erwinia herbicola* and *Pantoea citrea*. The folding prediction of G3DH suggested that the 3D structure of G3DH was similar with cholesterol oxidase from *Brevibacterium sterolicum* or glucose oxidase from *Aspergillus niger*.

Key Words: **glucose 3-dehydrogenase; marine bacteria; *Halomonas* sp.**

Various glucose oxidoreductases have been reported and utilized for the enzymatic determination of glucose. Among them, only few enzymes which oxidize hydroxy group of pyranose and its derivatives except C-1 position have been reported. Such enzymes can be applied for the production of sugar derivatives (1, 2) and the measurement of 1,5-anhydro-D-glucitol (1,5AG) (3–5), which is a major polyol in human blood and has been becoming a good clinical marker for diabetes. These include glucose 3-dehydrogenases (G3DHs) from *Agrobacterium tumefaciens* (6), *Flavobacterium saccharophilum* (7), *Cytophaga marinoflava* (8), *Agaricus bisporus* (9), and *Halomonas* (*Deleya* sp. α-15). However, only few works on these primary structure and cloning have been reported. The amino acid sequence of eight residues from the N terminus of G3DH from *Agaricus bisporus* is only the information about the primary structure of G3DHs (9). Schuerman *et al.* reported that introduction of *Agrobacterium tumefaciens* DNA fragment into *Escherichia coli* enabled *E. coli* to utilize aucrose as a sole carbon source. The DNA fragment of *A. tumefaciens* was suggested to contain G3DH structural gene, since this constructed recombinant *E. coli* produced 3-keto glucose (10). However, DNA sequencing and isolation of G3DH gene has not yet been reported.

We previously reported the isolation of a co-factor binding soluble G3DH which oxidizes 3rd hydroxy group of pyranose from a Gram-negative marine bacterium designated strain α-15, which was identified as *Deleya* sp. (11). Recently, genus *Deleya* was transferred to a genus *Halomonas* (12). Therefore, in this paper, we use the description *Halomonas* sp. α-15 instead of *Deleya* sp. α-15. This enzyme is a monomeric enzyme with M_r 63 kDa. This enzyme can oxidize monosaccharides like 1,5AG or α-methyl-D-glucoside and disaccharides like lactose or sucrose. On the other hand, the primary structure of G3DH is still unknown as same as other G3DH.

In this paper, we described the direct-expression cloning of G3DH structural gene from *Halomonas* sp. α-15. This is the first report of the enzyme structure which can oxidize hydroxy group of pyranose and its derivatives except C-1 position.

MATERIALS AND METHODS

Bacterial strain and culture media. *Halomonas* sp. α-15 (11) was used as the source of DNA in the gene-cloning experiment. *Escherichia coli* XL-1 Blue MR and *E. coli* DH5α were used as the host strains for direct-expression screening for the G3DH gene. *Halomonas* sp. α-15 was grown at 30°C in BSα medium, which contained polypepton (1% w/v), yeast extract (0.1% w/v), NaCl (3% w/v), KH_2PO_4 (0.2% w/v) and α-methyl-D-glucoside (0.4% w/v) or YM9Slac medium, which contained Na_2HPO_4 (0.6% w/v), KH_2PO_4 (0.3% w/v), NH_4Cl (0.1% w/v), yeast extract (0.02% w/v), $MgSO_4$ (0.02% w/v), $CaCl_2$ (0.002% w/v), NaCl (3% w/v) and lactose (0.8% w/v) as were described in the previous study. *E. coli* XL-1 Blue MR and *E. coli* DH5α were grown at 37°C in Luria broth medium.The cosmid used for cloning and expression of the G3DH gene in *E. coli* was SuperCos 1 (Staratagene, U.S.A.). The plasmid used for sequence and expression of the G3DH gene in *E. coli* was pUC18.

DNA preparation and manipulation. Total DNA from *Halomonas* sp. α-15 was prepared according to the manual of SuperCos 1

[1] To whom correspondence and reprint requests should be addressed. Fax: 81-42-388-7027. E-mail: sode@cc.tuat.ac.jp.

0006-291X/01 $35.00

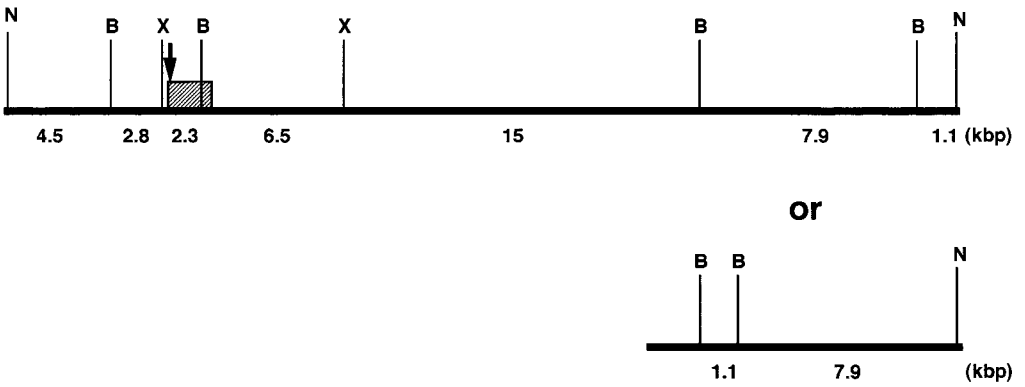

FIG. 1. Restriction map of clone 2. ↓, N-terminal of G3DH. ▨, G3DH structural gene. N, *Not*I; B, *Bam*HI; X, *Xba*I.

Cosmid Vector kit (Staratagene). General DNA manipulation were carried out as described by Sambrook *et al.* (13).

Construction of genomic library and screening of the G3DH gene. The total chromosomal DNA was partially digested with *Sau*3A I and the 30- to 42-kb DNA fragments were prepared. These fragments were ligated to SuperCos 1, which was linearized with *Xba*I and *Bam*HI and dephosphorylated with calf intestine alkaline phosphatase. The ligation mixture was packaged using the Gigapack III Gold Packaging Extract (Stratagene) and transduced to *E. coli* XL-1 Blue MR. The transformant colonies were selected using LB agar plate containing ampicillin and neomycin. These clones were cultivated in 3 ml LB medium containing 0.8% (w/v) lactose in tubes at 37°C overnight and whole cell G3DH assay was carried out.

Preparation of soluble fraction. Cells of *Halomonas* sp. α-15 or recombinant *E. coli* were harvested at the late exponential phase and washed three times with 10 mM potassium phosphate buffer (pH 7). The cells were disrupted by ultrasonication in 10 mM potassium phosphate buffer (pH 7). After centrifugation to remove the cell debris, the resulting supernatant was ultracentrifuged at 50000*g* for 60 min. The supernatant was designated as the soluble fraction.

Analytical method. Growth was monitored by measuring the optical density at 660 nm. The amount of G3DH production in *Halomonas* sp. α-15 and recombinant *E. coli* was measured as follows. One milliliter of culture was centrifuged for 5 min at 5000*g*. The centrifuged cells were washed with 10 mM potassium phosphate buffer (pH 7) three times and resuspended in 500 μl of 10 mM potassium phosphate buffer (pH 7). Ten microliters of the resuspended cells was used for whole cell G3DH assay. This sample was mixed with 80 μl of 10 mM potassium phosphate buffer (pH 7), 60 μM 2,6-dichlorophenol indophenol (DCIP) and 1 mM phenazine methosulfate (PMS) and measured the rate of absorbance decrease at 600 nm. G3DH assay of the soluble fraction was performed as follows. The soluble fraction of cells, 195 μl, was mixed with 2 μl of 1 M α-methyl-D-glucoside and 3 μl of 10 mM potassium phosphate buffer (pH 7), 4 mM DCIP and 66.7 mM PMS and measured the rate of absorbance decrease at 600 nm. Simultaneously, 30 μl of the resuspended cell sample was mixed with 10 μl of 10% SDS and boiled. Then, the protein concentration was measured using a DC protein assay kit (Bio-Rad, CA).

Purification, SDS–PAGE, and N-terminal amino acid sequence analysis. Purified G3DH was prepared as described previously (11). The prepared G3DH sample was subjected to SDS–PAGE separation and the band corresponding to G3DH was blotted to the PVDF membrane. This sample was used for the N-terminal amino acid sequence analysis by PPSQ-10 (Shimadzu, Japan).

DNA hybridization and sequencing. Southern hybridization was carried out at 40°C according to the manual of DIG DNA detection kit (Roche Diagnostics). The oligonucleotide probe used for hybridization was as follows: 5′-CCG GAC AAC CAC TAC GAC GCC ATC GTC GTC-3′. The sequence of this probe was designed based on the result of N-terminal amino acid sequence. The probe was labeled by DIG oligonucleotide tailing kit (Roche Diagnostic).

DNA sequence analysis of G3DH was performed in an automated DNA sequencer, ABI PRISM 310 genetic analyzer (Applied Biosystems, U.S.A.). Each region was sequenced at least three times.

RESULTS

N-Terminal Amino Acid Sequencing of G3DH

The G3DH of *Halomonas* sp. α-15 was purified from soluble fraction. The purified G3DH gave a band of about 63 kDa on SDS–PAGE. The N-terminal amino acid sequence of G3DH of *Halomonas* sp. α-15 was determined as NH_2-Pro-Asp-Asn-His-Tyr-Asp-Ala-Ile-Val-Val and no similarity was found to N-terminal amino acid sequence of G3DH from *Agaricus bisporus* (9).

Cloning and Expression of the Gene Encoding G3DH in E. coli

We tried to isolate the G3DH gene by a direct-expression method as described under Materials and Methods. Of 15 transformants, one putative positive clone was isolated and designated clone 2. Clone 2 showed dehydrogenase activity for glucose, α-methyl-D-glucoside and lactose in the presence of PMS-DCIP as the electron acceptor. The substrate specificity of dye mediated dehydrogenase activity was as same as *Halomonas* sp. α-15, indicating that G3DH had been cloned and expressed in *E. coli*. A recombinant cosmid containing about 40-kb DNA fragment was purified from the positive clone and designated pG3DH. The 40-kb fragment was excised by *Not*I and the restriction map of this fragment is obtained (Fig. 1). To identify the location of G3DH gene, this fragment was digested by *Bam*HI or *Xba*I and southern hybridization was carried out. The probe for G3DH N-terminal sequence was hybridized with a 5.1-kb *Bam*HI fragment and an 8.8-kb *Xba*I fragment. Therefore, G3DH gene was con-

TABLE 1

G3DH Activity in *Halomonas* sp. α-15 and *E. coli*

	G3DH activity (U/mg protein)
Halomonas sp. α-15	0.86
Clone 2	0.07
E. coli DH5α/pUCXba8.8kb	0.28
E. coli DH5α	0

tained in a 5.1-kb *Bam*HI fragment or an 8.8-kb *Xba*I fragment. These fragments were subcloned in pUC18 and designated pUCBam5.1kb and pUCXba8.8kb, respectively.

Next, the G3DH activity in recombinant *E. coli* was measured. The recombinant *E. coli* DH5α transformed with pUCXba8.8kb, *E. coli* XL-1 Blue MR harboring pG3DH and *Halomonas* sp. α-15 were cultivated in LB medium or YM9Slac medium. The soluble fraction was prepared and G3DH activity was measured. The results were shown in Table 1. The G3DH activity was found in the *E. coli* cells harboring pG3DH or pUCXba8.8kb although the G3DH activity in *E. coli* was lower than the activity in *Halomonas* sp. α-15. However, *E. coli* cell harboring pUCBam5.1kb did not show G3DH activity. These results indicated that the 8.8-kb *Xba*I fragment covered the whole region of G3DH gene.

Nucleotide Sequence of the G3DH Gene

The DNA sequence and deduced amino acid sequence of G3DH were determined and the results were shown in Fig. 2. The open reading frame (ORF) corresponding to G3DH might start with ATG at nucleotides 401 to 403, preceded by Shine–Dalgarno (SD) sequence, AAGGAAAA (nucleotides 388 to 495). The ORF terminate with TGA at nucleotides 2087 to 2089. A possible promoter sequence (−35, CTGACC at nucleotides 297 to 302, −10, AAAAAT at nucleotides 319 to 324) was found upstream of the G3DH gene. The gene consists of 1686 bp, encoding a polypeptide of 562 amino acid with a calculated molecular weight of 63172. The molecular mass of the polypeptide was in good agreement with the molecular mass obtained by SDS–PAGE (63 kDa). The N-terminal amino acid sequence of the purified G3DH was found in this ORF at positions 2 to 11. However, the methionine at position 1 was not found in the N-terminal amino acid sequencing analysis. This methionine residue may be processed by methionine aminopeptidase in *Halomonas* sp. α-15.

Comparison with Other Proteins

Comparison of G3DH polypeptide sequence to protein database using BLAST showed *Halomonas* sp. α-15 G3DH to be very similar to the large subunit of sorbitol dehydrogenase (SDH) from *Gluconobacter oxydans* and the catalytic subunit of 2-keto-D-gluconate dehydrogenases (2KGDH) from *Erwinia herbicola* (GenBank Accession No. AF068066) and from *Pantoea citrea* (14). The amino acid sequence alignment is shown in Fig. 3. The overall identity between G3DH and SDH or 2KGDH was about 30%. The similarity of G3DH to SDH and 2KGDH also extends to the predicted secondary structures using PHD (15–17). Similarly, the predicted locations of helices, sheets also show a remarkable resemblance between these enzymes.

The flavine adenine dinucleotide (FAD)-dependent enzymes, containing SDH and 2KGDH, possess the characteristic βαβ motif for binding the ADP moiety of FAD (19). This motif is usually located at the N-terminus of the enzyme and contains so-called glycine box (GXGXXG). The deduced amino acid sequence of G3DH contained glysine box, GSGISG, at positions 12 to 17. The secondary structure prediction using PHD also suggested that the N-terminal region around the glycine box possessed an βαβ structure.

The folding prediction of G3DH using 3D-PSSM (19, 20) suggested that the 3D structure of G3DH was similar with cholesterol oxidase (COD) from *Brevibacterium sterolicum* or glucose oxidase (GOD) from *Aspergillus niger*.

DISCUSSION

In this paper, we have cloned and expressed the gene encoding glucose 3-dehydrogenase (G3DH) from *Halomonas* sp. α-15 in *E. coli*. This is the first report of structure of glucose oxidoreductases which can oxidize hydroxy group of pyranose and its derivatives except C-1 position.

The deduced primary structure and the predicted secondary structure are similar to sorbitol dehydrogenase from *Gluconobacter oxidans* and 2-keto-D-gluconate dehydrogenases from *Erwinia herbicola* and *Pantoea citrea*. SDH from *G. oxydans* and 2KGDH from *E. herbicola* and *P. citrea* are composed with three subuints; catalytic subunit (about 65 kDa), cytochrome c subunit (about 40 kDa) and small subunit of which function is unknown (about 20 kDa). These enzymes exist as membrane-bound protein although G3DH was isolated from *Halomonas* sp. α-15 as a soluble enzyme. The primary structure similarity between G3DH and these enzyme may suggested that G3DH is also an oligomeric enzyme composed of catalytic subunit and electron transfer subunit. That G3DH coupled with respiratory chain is also consistent with this hypothesis. Further analysis of genetic construction of G3DH locus may elucidate these possible quaternary structure of G3DH.

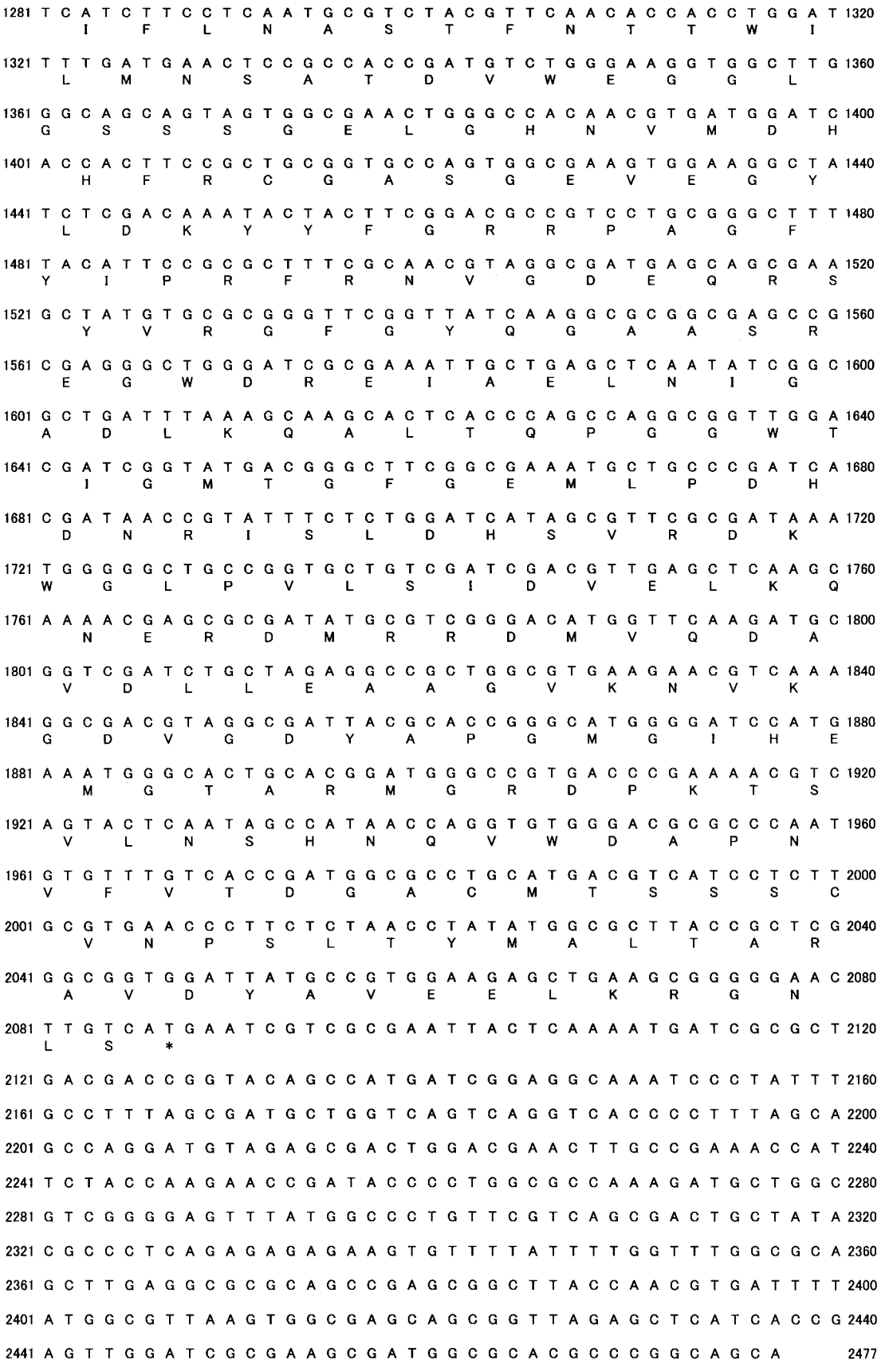

FIG. 2. DNA and amino acid sequence of G3DH from *Halomonas* sp. α-15. The underlined amino acid sequences were determined by N-terminal amino acid sequence. Potential promoter (-35 and -10 regions) and Shine–Dalgarno (SD) sequence are boxed.

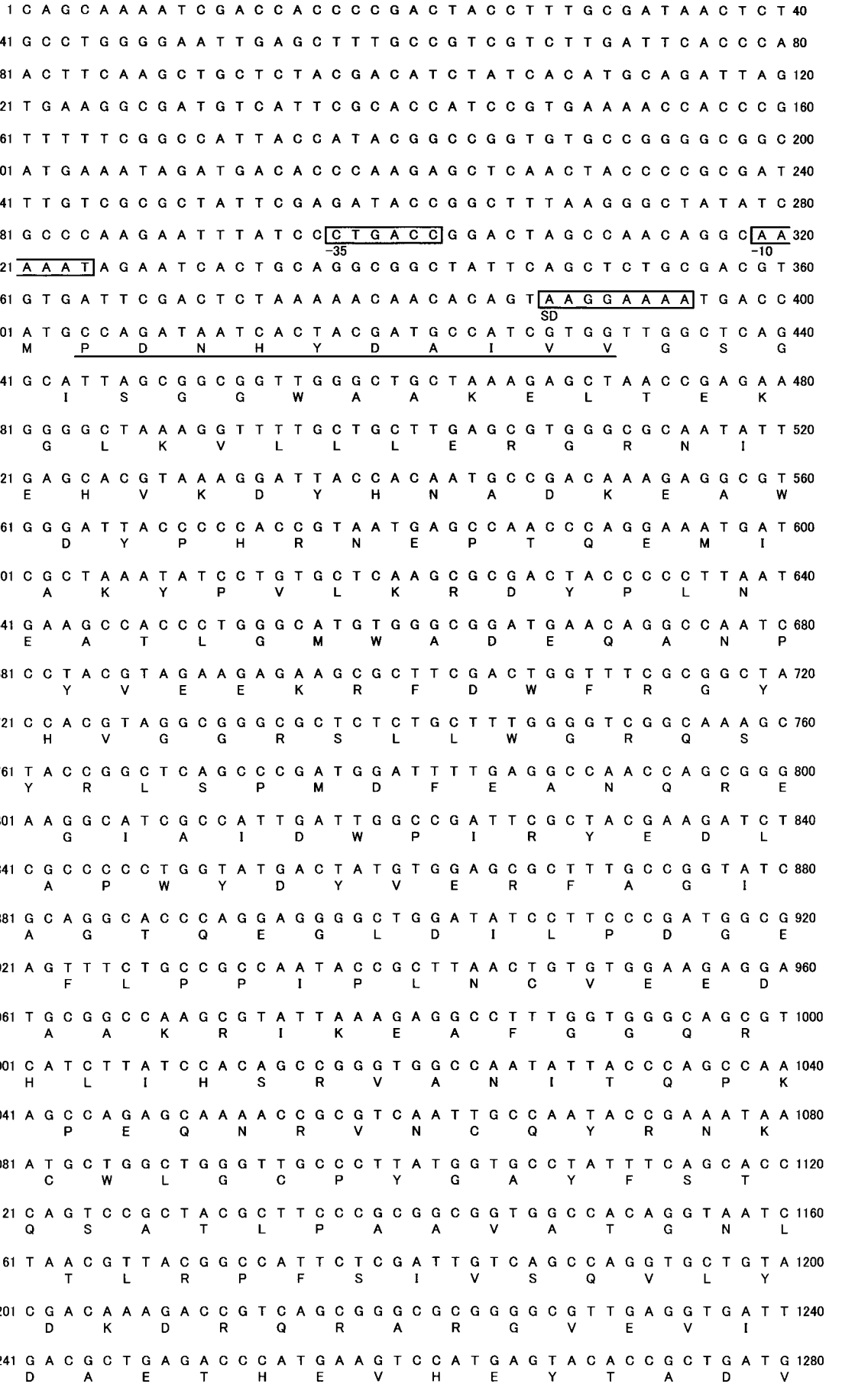

FIG. 2—*Continued*

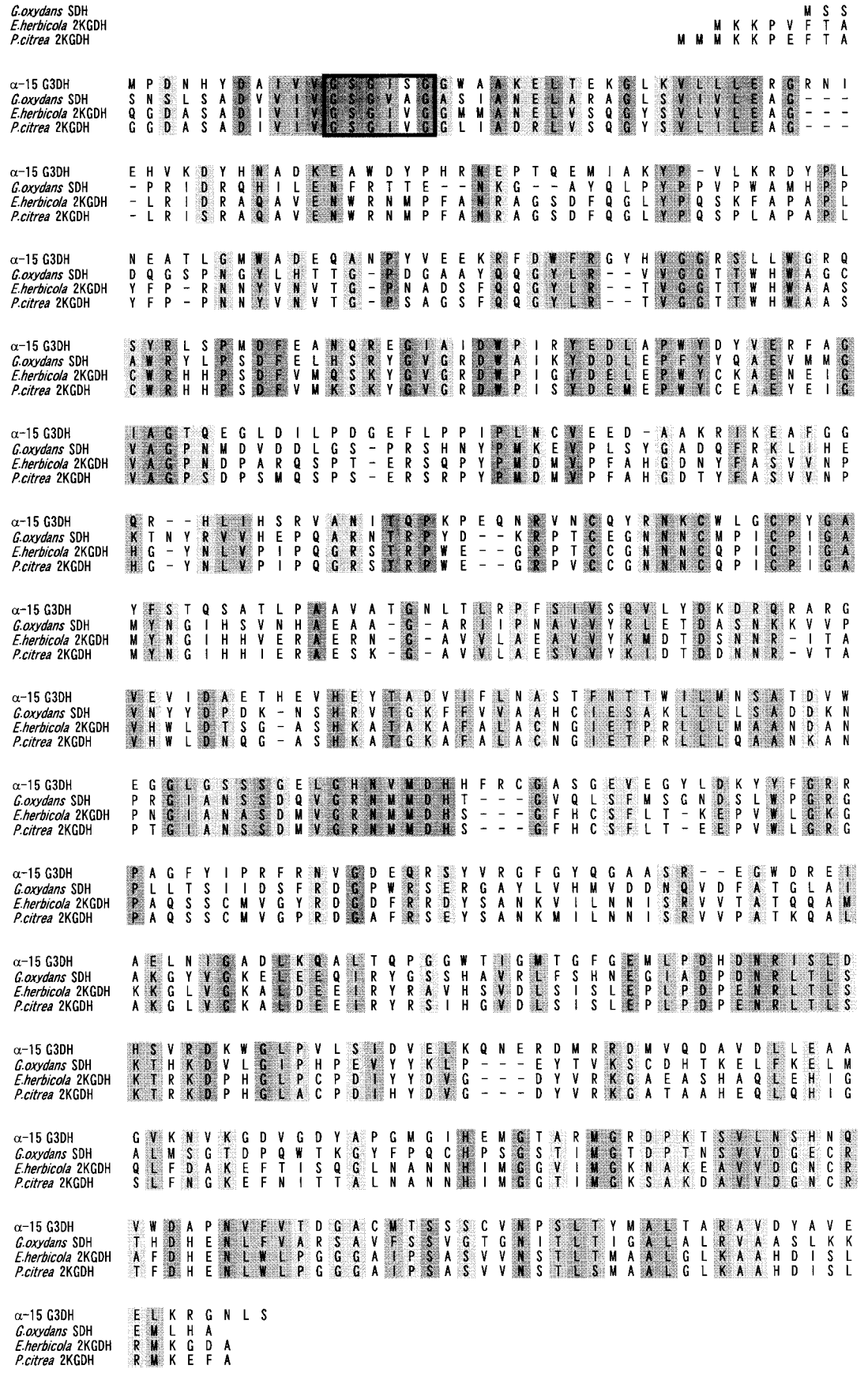

FIG. 3. Alignment of the amino acid sequences of G3DH with sorbitol dehydrogenase (SDH) and 2-keto-D-gluconate dehydrogenases (2KGDH). FAD binding region is boxed.

The folding prediction of G3DH suggested that the 3D structure of G3DH was similar with COD from *Brevibacterium sterolicum* or GOD from *A. niger*. The 3D structures and functional residues of COD and GOD have been reported (21, 22). The comparison of structural motifs between these enzymes and G3DH, the functional regions of responsible for catalytic activity and substrate recognition of G3DH will be revealed. These structure information should facilitate studying substrate specificity and substrate site specificity.

REFERENCES

1. Asano, N., Katayama, K., Takeuchi, M., Yoshikawa, M., and Matsui, K. (1989) Preparation of 3-amino-3-deoxy derivatives of trehalose and sucrose and their activities. *J. Antibiot. (Tokyo)* **42,** 585–590.
2. Stoppok, E., Matalla, K., and Buchholz, K. (1992) Microbial modification of sugars as building blocks for chemicals. *Appl. Microbiol. Biotechnol.* **36,** 604–610.
3. Yabuuchi, M., Masuda, M., Katoh, K., Nakamura, T., and Akanuma, H. (1989) Simple enzymatic method for determining 1,5-anhydro-D-glucitol in plasma for diagnosis of diabetes mellitus. *Clin. Chem.* **35,** 2039–2043.
4. Fukuyama, Y., Tajima, S., Oshitani, S., Ushijima, Y., Kobayashi, I., Hara, F., Yamamoto, S., and Yabuuchi, M. (1994) Fully enzymatic method for determining 1,5-anhydro-D-glucitol in serum. *Clin. Chem.* **40,** 2013–2016.
5. Tanabe, T., Umegae, Y., Koyashiki, Y., Kato, Y., Fukahori, K., Tajima, S., and Yabuuchi, M. (1994) Fully automated flow-injection system for quantifying 1,5-anhydro-D-glucitol in serum. *Clin. Chem.* **40,** 2006–2012.
6. Hayano, K., and Fukui, S. (1967) Purification and properties of 3-ketosucrose-forming enzyme from the cells of *Agrobacterium tumefaciens. J. Biol. Chem.* **242,** 3665–3672.
7. Takeuchi, M., Ninomiya, K., Kawabata, K., Asano, N., Kameda, Y., and Matsui, K. (1986) Purification and properties of glucoside 3-dehydrogenase from *Flavobacterium saccharophilum. J. Biochem. (Tokyo)* **100,** 1049–1055.
8. Tsugawa, W., Horiuchi, S., Tanaka, M., Wake, H., and Sode, K. (1996) Purification of marine bacterial glucose dehydrogenase from *Cytophaga marinoflava* and its application for measurement of 1,5-anhydro-D-glucitol. *Appl. Biochem. Biotechnol.* **56,** 301–310.
9. Morrison, S. C., Wood, D. A., and Wood, P. M. (1999) Characterization of glucose 3-dehydrogenase from the cultivated mushroom (*Agaricus bisporus*). *Appl. Microbiol. Biotechnol.* **51,** 58–64.
10. Schuerman, P. L., Liu, J. S., Mou H., and Dandekar, A. M. (1997) 3-Ketoglycoside-mediated metabolism of sucrose in *E. coli* as conferred by genes from *Agrobacterium tumefaciens. Appl. Microbiol. Biotechnol.* **47,** 560–565.
11. Tsugawa, W., Ogasawara, N., and Sode, K. (1998) Fluorescent measurement of 1,5-anhydro-D-glucitol based on a novel marine bacterial glucose dehydrogenase. *Enzyme Microbial Technol.* **22,** 269–274.
12. Dobson, S. J., and Franzmann, P. D. (1996) Unification of the genera Deleya (Baumann *et al.,* 1983), Halomonas (Vreeland *et al.*, 1980), and Halovibrio (Fendrich, 1988) and the species *Paracoccus halodenitrificans* (Robinson and Gibbons, 1952) into a single genus, Halomonas, and placement of the genus Zymobacter in the family Halomonadaceae. *Int. J. Syst. Bacteriol.* **46,** 550–558.
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Springer Harbor Laboratory Press, Cold Springer Harbor, NY.
14. Pujol, C. J., and Kado, C. I. (2000) Genetic and biochemical characterization of the pathway in *Pantoea citrea* leading to pink disease of pineapple. *J. Bacteriol.* **182,** 2230–2237.
15. Rost, B., and Sander, C. (1993) Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.* **232,** 584–599.
16. Rost, B., and Sander, C. (1994) Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins* **19,** 55–77.
17. Rost, B. (1996) PHD: Predicting one-dimensional protein structure by profile based neural networks. *Methods Enzymol.* **266,** 525–539.
18. Wierenga, R. K., Terpstra, P., and Hol, W. G. J. (1986) Prediction of the occurrence of the ADP-binding beta alpha beta-fold in proteins, using an amino acid sequence fingerprint. *J. Mol. Biol.* **187,** 101–107.
19. Fischer, D., Barret, C., Bryson, K., Elofsson, A., Godzik, A., Jones, D., Karplus, K. J., Kelley, L. A., Maccallum, R. M., Pawowski, K., Rost, B., Rychlewski, L., and Sternberg, M. J. (1999) CAFASP-1: Critical assessment of fully automated structure prediction methods. *Proteins* Suppl. 3, 209–217.
20. Kelley, L. A., Maccallum, R., and Sternberg M. J. E. (1999) Recognition of remote protein homologies using three-dimensional information to generate a position specific scoring matrix in the program 3D-PSSM. *In* RECOMB 99, Proceedings of the Third Annual Conference on Computational Molecular Biology (Istrail, S., Pevzner, P., and Waterman, M., Eds.), pp. 218–225, Association for Computing Machinery, New York.
21. Vrielink, A., Lloyd, L. F., and Blow, D. M. (1991) Crystal structure of cholesterol oxidase from *Brevibacterium sterolicum* refined at 1.8 Å resolution. *J. Mol. Biol.* **219,** 533–554.
22. Hecht, H. J., Kalisz, H. M., Hendle, J., Schmid, R. D., and Schomburg, D. (1993) Crystal structure of glucose oxidase from *Aspergillus niger* refined at 2.3 Å resolution. *J. Mol. Biol.* **229,** 153–172.