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Cloning of the Gene Encoding Quinoprotein Glucose Dehydrogenase from *Acinetobacter calcoaceticus*: Evidence for the Presence of a Second Enzyme

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We cloned the gene coding for the quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus*. This clone complements *gdh* mutations in *A. calcoaceticus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The gene codes for a protein with an M_r of 83,000. Evidence is presented for the presence of two different glucose dehydrogenase enzymes in *A. calcoaceticus*: a protein with an M_r of 83,000 and a dimer of two identical subunits with an M_r of 50,000.

The quinoprotein glucose dehydrogenase (GDH) (EC 1.1.99.17) is a membrane-bound enzyme which converts glucose and other aldoses to their corresponding acids (10). In this reaction pyrroloquinoline-quinone (PQQ) is used as a coenzyme (6). PQQ-dependent GDH has been detected in a wide variety of bacterial species, including *Gluconobacter suboxydans* (2), *Acinetobacter calcoaceticus* (10), *Klebsiella aerogenes* (19), *Pseudomonas aeruginosa* (17), and *Escherichia coli* (11). In most of the bacteria the GDH holoenzyme is synthesized. In *E. coli* (11), *Klebsiella pneumoniae* (19), and *Acinetobacter lwoffi* (22), however, only the apoenzyme is formed, which becomes active on the addition of PQQ to the growth medium.

It has been shown that a GDH protein is located at the periplasmic side of the cytoplasmic membrane (15, 17). Therefore, the product of the GDH reaction is formed outside the cell. In most of the GDH-containing bacteria, this reaction product (gluconic acid, when glucose is used) can subsequently be transported into the cell and used as a carbon source. In most *A. calcoaceticus* strains, however, gluconic acid cannot be metabolized further. Therefore, these strains are not able to grow on glucose as the sole carbon source, but physiological studies have shown that the dehydrogenase reaction is associated with ATP synthesis and generates a proton motive force, which can drive the secondary transport of amino acids and lactose (21, 21a). L-Arabonic acid, however, the GDH product of L-arabinose, can be fully metabolized and used as a carbon source by *A. calcoaceticus* LMD79.41 (9). An *A. calcoaceticus* strain that is mutated either in its PQQ production or its GDH function is no longer able to grow on L-arabinose as the sole carbon source.

GDH was isolated from different bacterial species, and the properties were studied. Dokter et al. (4) have reported the purification of GDH from *A. calcoaceticus*. The molecular weight was estimated to be 94,000, and it was shown that the enzyme consists of two identical subunits with an M_r of approximately 48,000. Independently, Geiger and Görisch (7) isolated GDH from *A. calcoaceticus* and showed that it consists of two subunits with an estimated molecular weight of 54,000. Purification of GDHs from *Pseudomonas fluorescens* (14), *G. suboxydans* (2), and *E. coli* (1) has also been reported. The enzymes from these organisms have molecular weights ranging from 83,000 to 88,000 under denaturing conditions. Antibodies raised against purified GDH from *P. fluorescens* showed cross-reactivity with crude membranes of *G. suboxydans*, *K. pneumoniae*, *Acetobacter aceti*, *P. aeruginosa*, and *E. coli* (15), suggesting that the GDH enzymes from these bacterial species are closely related.

Besides the difference in molecular weights, the GDHs isolated from *Acinetobacter calcoaceticus* (4, 7) and *E. coli* (1) also showed differences in isoelectric point and substrate specificity. However, immunoblotting showed that antibodies directed against purified GDH of *P. fluorescens* also reacted with a protein with an M_r of 83,000 from *A. calcoaceticus* (15), which is inconsistent with the molecular weight of 50,000 found for the purified *A. calcoaceticus* enzyme. In this report we describe the isolation of the *gdh* gene from *A. calcoaceticus* by complementation of GDH$^-$ mutants. We show that this *gdh* gene synthesizes a protein with an M_r of 83,000 which can also complement GDH mutants of *E. coli*. Our results suggest the presence of two GDH enzymes, which would explain the contradictory results reported in the literature.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli*, *A. calcoaceticus*, and *Pseudomonas* strains were cultured in L broth, on L-agar plates, or in defined minimal medium as indicated previously (18). Plasmids were transformed to *E. coli* as described previously (13). Tetracycline was used at a concentration of 20 μg/ml, kanamycin at 50 μg/ml, and ampicillin at 40 μg/ml for all strains.

Chemicals and reagents. All restriction enzymes, T4 DNA ligase, the Klenow fragment of DNA polymerase I, and calf intestine phosphatase were obtained commercially. Purified PQQ and Würster blue, an electron acceptor dye, made by one-electron oxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, were gifts from M. van Kleef. PQQ was used at a concentration of 2 μM for *A. calcoaceticus* and 12 μM for *E. coli* and *Pseudomonas stutzeri*.

Analysis of plasmid DNA. Plasmid DNA from *E. coli* was isolated as described by Maniatis et al. (13). Electrophoresis of DNA fragments was carried out on 1% agarose gels in 40 mM Tris acetate–2 mM EDTA (pH 8).

Bacterial matings. The pLV21 derivatives of the genomic bank were transferred to *A. calcoaceticus* with the helper

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TABLE 1. Strains and plasmids used in this study

Strains and plasmids	Genotype or phenotype	Source or reference
Escherichia coli		
MH1	*araD lacX74 galU hsdR hsdM rpsL*	8
JM101	*lac pro supE thi* F::*traD36 proAB lacI*^{q} *lacZ*ΔM15	16
S17-1	RP4 derivative integrated into the chromosome	20
PP1192	*thi*Δ(*pro-lac*) *supE rpsL* Fts*lac*::Tn*5*	Our laboratory
PPA42	*ptsI thi galP*	P. Postma
PP1795	*ptsI thi galP gdh*	This work
Acinetobacter calcoaceticus		
LMD79.41	Wild type	6
PP1362-PP1376	*gdh*	This work
Pseudomonas aeruginosa 2F32-106B	*gdh*	17
Pseudomonas stutzeri LMD26.38	Wild type	M. van Kleef
Plasmids		
pUC12	Ap^r	23
pUC19	Ap^r	23
pLV21	Su^r Km^r; hybrid plasmid of RSF1010 and pKB110	P. T. Barth
RP4 ΔKm	Ap^r Tc^r; helper plasmid to mobilize pLV21	Our laboratory
pRK290	Tc^r	3
pGP426	Tc^r; pRK290 derivative containing the *gdh* gene	This work
pGP468	Ap^r; pUC19 derivative containing the *gdh* gene	This work
pGP469	Ap^r; pUC12 derivative containing the *gdh* gene	This work

plasmid RP4ΔKm, as described previously (9). For the conjugation of pRK290 derivatives, the *E. coli* strain S17-1 was used, which carries a derivative of the helper plasmid RP4 in the chromosome (20). Transconjugants of *A. calcoaceticus* or *Pseudomonas* species were selected by plating on minimal medium containing citric acid and tetracycline.

Assay for PQQ production. To test whether the GDH$^-$ mutants isolated still produced PQQ, the strains were mixed 1:1 with PP1335, a PQQ mutant of *A. calcoaceticus* (9), and plated onto MacConkey agar containing 25 mM glucose. Complementation of the PQQ$^-$ mutant by PQQ produced by the GDH$^-$ mutant resulted in red colonies.

Isolation of GDH$^-$ mutants in *E. coli*. *E. coli* PPA42 was grown in L broth to a density of 5×10^8 cells per ml. The cells were washed, and the pellet was suspended in minimal medium containing 10 μM thiamine and 50 mM gluconic acid at a concentration of 5×10^9 cells per ml. Ethyl methanesulfonate was added to a concentration of 40 μg/ml. After incubation at 37°C for 30 min, the cells were washed, suspended in L broth, and grown overnight. Subsequently, the cells were plated onto eosin-methylene blue agar (Difco Laboratories, Detroit, Mich.) containing 30 mM glucose and 6 μM PQQ. Non-acid-producing colonies (which could be identified, because their color was less purple-red than the wild type) were tested on minimal medium. GDH$^-$ mutants were no longer able to grow on minimal medium with glucose and PQQ. Growth on gluconic acid was still possible.

In vitro transcription translation. A commercially available kit (procaryotic DNA-directed translation kit; Amersham Corp., Arlington Heights, Ill.) was used to show the size of the protein product from the GDH clone. About 1 μg of CsCl-purified plasmid DNA was used. The reaction products were electrophoresed on a 12% polyacrylamide gel containing sodium dodecyl sulfate (12). ^{35}S-labeled protein products were visualized by autoradiography.

Preparation of cell extracts from *A. calcoaceticus* and GDH enzyme assay. Cell extract was prepared from cells and grown in L broth, as described previously (6). GDH activities were determined at room temperature in a mixture of 0.1 M Tris hydrochloride (pH 7)–0.1 mg of Würster blue per ml–40 mM D-glucose–20 μl of cell extract. GDH activity was determined by measuring the decrease in the A_{610}. Calculations of the GDH activity were based on the molar extinction coefficient for Würster blue: 12,400 M^{-1} cm^{-1} at 610 nm (4).

RESULTS

Cloning of the *gdh* gene of *A. calcoaceticus*. The isolation of *A. calcoaceticus* mutants that no longer produce acid on medium containing glucose has been described previously (9). These mutants, which were also deficient in growth on arabinose as the sole carbon source, were divided into two classes. The first class of mutants could be complemented for acid production by the addition of purified PQQ to the medium and was shown to contain a mutation in one of the PQQ genes (9). The second class of mutants was not affected in PQQ synthesis, since production of PQQ in the culture medium could be detected (see above). Therefore, these strains probably contain mutations in their *gdh* genes. We isolated 15 independent GDH$^-$ mutants (Table 1).

For the isolation of the *gdh* gene, we used the genomic colony bank of *A. calcoaceticus* in the broad-host-range vector pLV21 described by Goosen et al. (9). The colony bank was transferred to one of the GDH$^-$ mutants, as described previously (9). Transconjugants were tested for complementation of their GDH activities on minimal medium containing arabinose. In this way a plasmid (p5.10F) was found that complemented all 15 GDH$^-$ mutants for growth on arabinose. Next, the 13.3-kilobase (kb) insert of p5.10F was partially digested with *Sau*3A, and the *Sau*3A fragments were randomly inserted into the *Bgl*II site of the vector pRK290 (3). This resulted in the subclone pGP426, which carried an insert of 3 kb and which still complemented all GDH$^-$ mutants.

To map the *gdh* gene in pGP426, Tn*5* insertions were isolated as described previously (9). The insertions were mapped by restriction enzyme analysis and tested for complementation of the *gdh* mutation (Fig. 1). The *gdh* operon

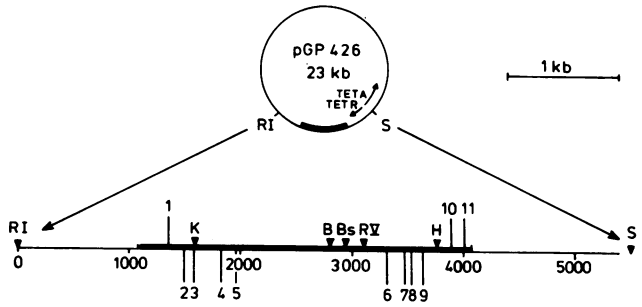

FIG. 1. Restriction map of the insert of pGP426. Restriction enzyme abbreviations: B, *Bam*HI; Bs, *Bst*EII; H, *Hin*dIII; K, *Kpn*I; RI, *Eco*RI; RV, *Eco*RV; S, *Sal*I. The positions of the different Tn*5* insertions are indicated with vertical lines. Lines pointing down indicate insertion mutations within the gene, because they can no longer complement a GDH$^-$ mutant. Lines pointing up indicate that the insertion mutations had no effect on complementation.

appears to be about 2.4 kb in size. This region can code for a protein with a maximum molecular weight of approximately 86,000.

Expression of the *gdh* gene in *Pseudomonas* species. We studied the expression of the *A. calcoaceticus gdh* gene in two different *Pseudomonas* strains. The first strain is *P. aeruginosa* 2F32-106B, which has a mutation in *gdh* (5). The second strain is *P. stutzeri* LMD26.48, which naturally lacks the GDH enzyme (M. van Kleef, personal communication). pGP426 was introduced into both strains by conjugation, and subsequently, these transconjugants were tested for acid production on MacConkey agar containing different GDH sugars (Table 2). The acid production of *P. aeruginosa* 2F32-106B was complemented by our clone. Also, *P. stutzeri* was able to perform the GDH reaction, provided that PQQ was added to reconstitute the holoenzyme. Colonies grown on MacConkey agar containing glucose were red, even without the *gdh* clone, because *Pseudomonas* codes for another glucose-metabolizing system (17).

GDH$^-$ mutants of *E. coli*. To determine whether the *A. calcoaceticus gdh* gene is expressed in *E. coli*, we isolated GDH$^-$ mutants in *E. coli* PPA42 (kindly provided by P. Postma, University of Amsterdam, Amsterdam, The Netherlands). This is an *E. coli* K-12 strain that lacks enzyme I of the phosphoenolpyruvate phosphotransferase system (11) but which still synthesizes the GDH apoenzyme. Growth on glucose as the sole carbon source is possible when PPA42 cells are grown in the presence of PQQ, which reconstitutes the GDH holoenzyme. Four independent *pts gdh* double mutants were isolated, as described above. These mutants were able to grow on gluconic acid, but not on glucose with or without PQQ.

TABLE 3. Growth on glucose by *E. coli gdh* mutants with or without a *gdh* plasmid

Mutant	Growth on[a]:		
	D-(+)-Glucose	D-(+)-Glucose with PQQ	D-(+)-Gluconic acid
PPA42	−	++	+++
PP1795	−	−	+++
PP1795(pGP426)	−	+	+++
PP1795(pRK290)	−	−	+++
PP1795(pGP468)	−	++	+++
PP1795(pGP469)	−	−	−
PP1795(pUC19)	−	−	+++

[a] Symbols: −, no growth; +, moderate growth; ++, intermediate growth; +++, good growth.

The *gdh* plasmid pGP426 was brought into one of the *E. coli* GDH$^-$ mutants (PP1795) by transformation. A transformant was tested for complementation of its GDH function on minimal medium containing gluconic acid, glucose with PQQ, or glucose alone (Table 3). Growth on glucose and PQQ was observed, which implies that the *A. calcoaceticus gdh* clone can complement the GDH$^-$ mutation in *E. coli*. The growth rate, however, was not as high as that on gluconic acid. Similar results were obtained when pGP426 was introduced into the other *E. coli* GDH$^-$ mutants (data not shown).

The low level of complementation could have been due to the suboptimal expression from the *A. calcoaceticus gdh* promoter in *E. coli*. To test this the complementing sequence was cloned in pUC12 and pUC19, respectively, in two orientations downstream of the *E. coli lac* promoter. The construction of the plasmids pGP468 and pGP469 is presented in Fig. 2.

On induction of the *lac* promoter by isopropyl-β-D-thiogalactopyranoside, there was a marked decrease in the growth rate of *E. coli* JM101(pGP469). No interference with growth rate could be observed with pGP468 in JM101. Apparently, in pGP469 the *gdh* gene was transcribed under the control of the *lac* promoter, leading to overproduction of GDH and the subsequent inhibition of cell growth. Preliminary sequence results support this conclusion.

Next, we transformed PP1795 with pGP469 to test complementation of the *gdh* mutation by this plasmid. Due to the relatively high expression of *gdh* from pGP469 in PP1795, the cells grew very poorly and failed to grow at all on minimal medium, so complementation could not be tested. Plasmid pGP468, which contained the *gdh* gene under the control of its own promoter (Fig. 2), restored PP1795 for growth on glucose and PQQ (Table 3). The growth rate was somewhat higher than that in PP1795 carrying pGP426. This

TABLE 2. Acid production of *P. aeruginosa* 2F32-106B and *P. stutzeri* LMD26.38 with or without pGP426 on MacConkey agar with different aldoses

Substrate	Acid production by:					
	2F32-106B	2F32-106B (pGP426)	LMD26.38		LMD26.38(pGP426)	
			Without PQQ	With PQQ	Without PQQ	With PQQ
D-(+)-Glucose	+	+	+	+	+	+
L-(+)-Arabinose	−	+	−	−	−	+
D-(+)-Xylose	−	+	−	−	−	+
D-(+)-Galactose	−	+	−	−	−	+
Lactose	−	−	−	−	−	−

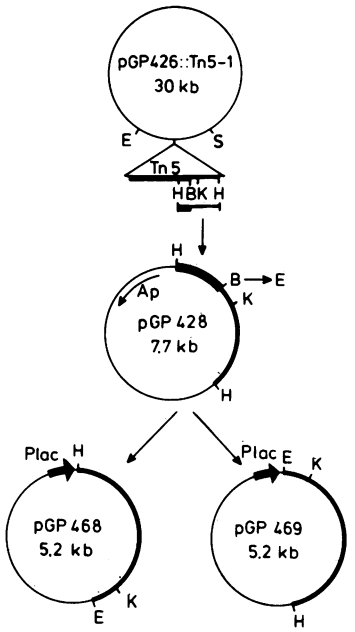

FIG. 2. Construction of pGP468 and pGP469. Plasmid pGP426 carrying Tn*5* insertion 1 (Fig. 1) was digested with *Hin*dIII. This generated a 3.4-kb fragment containing 1 kb of one inverted repeat of Tn*5* and 2.4 kb with the *gdh* gene. The vector pBR322 was digested with *Hin*dIII and subsequently treated with calf intestine phosphatase. The 3.4-kb *Hin*dIII fragment was ligated into the *Hin*dIII site of pBR322. The resulting recombinant plasmid was digested with *Bgl*I. Protruding ends were filled with Klenow fragment polymerase I, and subsequently phosphorylated *Eco*RI linkers were ligated to the blunt ends. After digestion with *Eco*RI and *Hin*dIII, the 2.4-kb *Eco*RI-*Hin*dIII fragment containing the *gdh* gene was isolated with low-melting-point agarose and ligated into the polylinkers of pUC12 and pUC19. This insert contained only 80 base pairs from Tn*5*. Restriction enzyme abbreviations: E, *Eco*RI; S, *Sal*I; H, *Hin*dIII; B, *Bgl*I; K, *Kpn*I.

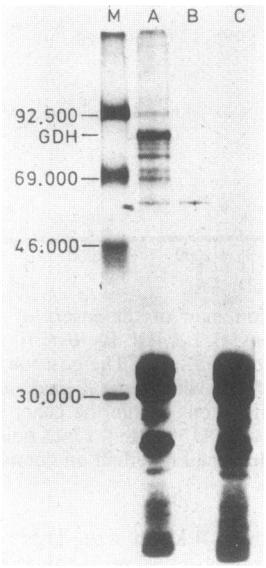

FIG. 3. In vitro transcription-translation of pGP469. Lane M, Molecular weight markers; lane A, pGP469; lane B, no DNA; lane C, pUC12. The size of GDH transcribed from pGP469 was estimated as 83,000.

was probably due to the high copy number of pUC19, the vector of recombinant plasmid pGP468, which would compensate for the low efficiency of the *Acinetobacter* promoter. In summary, the *gdh* gene of *A. calcoaceticus* is able to complement a GDH mutant in *E. coli*.

The *gdh* gene codes for a protein with an M_r of 83,000. To define the size of the protein product of the cloned *gdh* gene, we used a commercially available in vitro transcription-translation kit. This kit contained extracts of *E. coli*, and therefore, pGP469, the plasmid with the *gdh* gene under control of the *lac* promoter, was tested in this reaction. The *gdh* clone coded for one protein with an M_r of about 83,000 (Fig. 3).

Evidence for the presence of two different GDH enzymes. The results presented in Fig. 3 are consistent with the results of Matsushita et al. (15), who have shown by immunoblotting that a protein of *A. calcoaceticus* with an M_r of 83,000 shows cross-reactivity with an antiserum directed against purified GDH of *P. fluorescens*. Other GDH enzymes that have been purified from *E. coli* (1), *P. fluorescens* (14), and *G. suboxydans* (2) also have a PQQ-dependent GDH in the same size range. However, both Dokter et al. (4) and Geiger and Görisch (7) showed that purified GDH from *A. calcoaceticus* consists of two identical monomers with a molecular weight of approximately 50,000 (48,000 according to Dokter et al. [4] and 54,000 according to Geiger and Görisch [7]). These observations suggest that *A. calcoaceticus* has two different enzymes with PQQ-dependent GDH activities.

To test the hypothesis of the presence of two GDH genes, we isolated cell extracts of wild-type *A. calcoaceticus* LMD 79.41 and two different GDH$^-$ mutants and measured the enzyme activities, as described above. The extract of LMD 79.41 contained a GDH activity of 2.56 units per ml. In the GDH$^-$ mutants PP1367 and PP1369, activities of 1.31 and 1.60 units per ml, respectively, were measured. These results indicate that, although the GDH$^-$ mutants are deficient in glucose oxidation in vivo, they still show considerable GDH activity in vitro. Moreover, it was possible to isolate a protein with an M_r of 50,000 with GDH activity from the extract of one of the mutants (PP1367) (P. Dokter, personal communication). These results indicate that *A. calcoaceticus* codes for two different GDH enzymes, one with an M_r of 83,000 and one with an M_r of approximately 50,000.

DISCUSSION

In this report we described the isolation of the gene coding for the PQQ-dependent GDH of *A. calcoaceticus*. The *gdh* gene codes for a protein with an M_r of approximately 83,000. This is in agreement with the results published by Matsushita et al. (15), who showed that antibodies raised against purified GDH of *P. fluorescens* reacted with a protein from *A. calcoaceticus* with an M_r of 83,000. The cloned *gdh* gene complemented GDH$^-$ mutants of *A. calcoaceticus* and *P. aeruginosa*. Moreover, on introduction of the *gdh* gene into *P. stutzeri* or GDH$^-$ mutants of *E. coli* the GDH apoenzyme was synthesized; this enzyme could be functionally activated by the addition of PQQ to the growth medium.

Dokter et al. (4) and Geiger and Görisch (7) have described the isolation of an enzyme from *A. calcoaceticus* which has PQQ-dependent GDH activity in vitro. This enzyme is a dimer of two identical subunits. However, the molecular weight of the subunits was determined to be about 50,000, which differs considerably from the size of the cloned GDH enzyme. This led us to the hypothesis that there are two

different GDH enzymes in *A. calcoaceticus*. Indeed, in the cell extracts prepared from the GDH$^-$ mutants of *A. calcoaceticus* with an M_r of 50,000, in vitro GDH activity could still be detected.

The GDH enzyme with an M_r of 50,000 did not seem to be functional in intact cells, as none of the GDH$^-$ mutants isolated showed glucose oxidation in vivo. Only after subsequent lysis of the cells, solubilization of the cell membranes with Triton X-100, and addition of the artificial electron acceptor Würster blue could activity of the protein with an M_r of 50,000 be detected.

The supposed presence of two different GDH enzymes also explains the difference observed between in vivo and in vitro substrate specificities. The purified GDH with an M_r of 50,000 showed oxidation of the disaccharides lactose, maltose, and cellobiose. These disaccharides were not oxidized in vivo by whole cells. This was not due to the presence of the outer membrane, which might act as a barrier to the larger disaccharide molecules, because membrane vesicles (lacking the outer membrane) also do not oxidize lactose (5). After solubilization of the vesicles and in the presence of phenazine methosulfate as the electron mediator, oxidation of lactose was observed. From these results Dokter et al. (5) concluded that GDH cannot act on the large disaccharide molecules when it is anchored in the membrane.

As we know now that two different GDH enzymes might be present in *A. calcoaceticus*, it is much more likely that the GDH with an M_r of 83,000 (which seems to be the only GDH that is active in whole cells and membrane vesicles) cannot oxidize disaccharides, whereas the enzyme with an M_r of 50,000 (which is supposed to be active on solubilization, in the presence of an electron mediator) can. This hypothesis is supported by the observation that GDH from *E. coli*, which cross-reacts with the same antibodies as the GDH with an M_r of 83,000 from *A. calcoaceticus* (15), also is not able to oxidize lactose in vitro (1). The GDH with an M_r of 50,000 might be unique in *A. calcoaceticus*, whereas the form with an M_r of 83,000 is widespread among many bacterial species, as indicated by immunological data (15). The question remains as to what the biological role for the enzyme with an M_r of 50,000 might be in *A. calcoaceticus*, since it could not react in vivo with the aldoses that were present in the culture medium. One possibility that can be considered is that the GDH with an M_r of 50,000 plays a role in the degradation of aldoses and disaccharides that have been metabolized in the cell. Experiments to study the precise role of the GDH with an M_r of 50,000 are currently under way.

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