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Analysis of L-glucose metabolic pathway in *Paracoccus* sp. 43P[1)]

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Research aims

Almost all the living organisms on the Earth can utilize D-glucose as carbon and energy sources, and its metabolic pathways are known in detail, such as the glycolysis and the pentose-phosphate pathway. On the other hand, L-glucose, the enantiomer of D-glucose, is thought not to be present in the natural environment, and any L-glucose-utilizing organisms are not known. Rather, since Rudney[2)] reported in 1940 that some bacteria, yeast and mammals did not utilize it, it has been generally accepted that there are no organisms that can utilize it. Therefore it can be assumed that there is the homochirality of certain sugar molecules, especially for glucose, as in the case of the homochirality of L-amino acids in proteins.

To challenge this "homochirality in sugar metabolism", we have screened for microorganisms which could utilize L-glucose from natural environments, and succeeded in obtaining such a bacterium, termed *Paracoccus* sp. 43P. We also revealed that the first step for L-glucose utilization was the reaction catalyzed by an NAD^+-dependent L-glucose dehydrogenase (L-GDH).

In this work, we have analyzed the enzymes and corresponding genes involved in L-glucose metabolism and succeeded in clarifying the metabolic pathway for L-glucose utilization in this bacterium.

Methods

Sequence analysis of the region around lgdA, encoding L-GDH, in strain 43P.

The DNA fragments of the region surrounding *lgdA*, which was already cloned and sequenced, were PCR-amplified by the primer-walking method from the genome of strain 43P, and their nucleotide sequences were determined.

Production and purification of the enzymes involved in L-glucose metabolism.

The putative ORFs were PCR-amplified and cloned into pET21a or pET28a, with the 6xHis-tag in the plasmid fused in-frame with each ORF at its C- or N-terminus, respectively, and the protein products were produced in *Escherichia coli* BL21(DE3) harboring their respective plasmids. The enzymes were purified by an affinity chromatography against the 6x His-tag.

Analyses of the enzyme activities.

The purified enzymes were used to detect their presumed enzyme activities under the conditions described previously. The substrates used were commercially available sugars and aldonates, or their derivatives made by chemical and/or enzymatic reactions.

Identification of the reaction products.

Some of the reaction products were purified by TLC, and identified by HPLC analysis. GC/MS or ^{1}H-NMR analysis was also conducted to identify the products.

Results

Sequencing analysis of the region around lgdA.

The amino acid sequence of the cloned *lgdA* from strain 43P, encoding the L-GDH, showed 84% identity with that of Pden_1680 from *P. denitrificans* PD1222, and the Pden_1680 gene was located in a cluster of genes annotated as involved in inositol metabolism. Sequencing analysis of the region around *lgdA* in strain 43P showed the same gene organization as that of strain PD1222, with amino acid identities of 76–93%, suggesting that the LgdA might have a functional role in inositol metabolism (Fig. 1). In fact, the purified L-GDH showed dehydrogenase activity against several inositols. Especially, the activity against *scyllo*-inositol (190 U/mg protein) was about six times-higher than that against L-glucose (31 U/mg protein), suggesting that the substrate of this enzyme was *scyllo*-inositol under the physiological conditions.

The reaction product generated from L-glucose by the action of L-GDH was identified as L-glucono-1,5-lactone by GC/MS analysis, indicating that L-GDH catalyses oxidation of L-glucose at C1 position. This compound is known to be unstable in an aqueous solution and is easily hydrolyzed to L-gluconate.

Cloning of L-GnDH gene and sequencing analysis of the region around the gene.

Next, we searched for an enzyme activity against L-gluconate, which was synthesized from L-glucose by I_2 oxidation, in the cell-free extract of strain 43P, and detected an NAD^+-dependent L-gluconate dehydrogenase activity. The enzyme was purified and its N-terminal amino acid sequence was determined. The sequence coincided with that of Pden_4931 of strain PD1222, indicating that the L-GnDH is encoded by the orthologous gene of Pden_4931 in

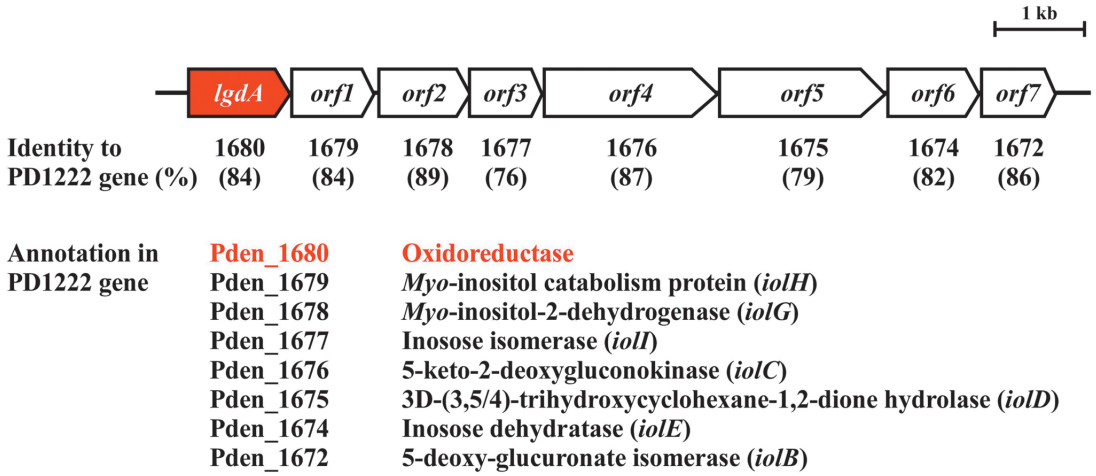

Fig. 1. Gene organization of the region around *lgdA* gene in the genome of strain 43P. The ORFs are shown with boxes, and the amino acid sequence identity of each ORF with the corresponding gene from strain PD1222 is indicated. Annotations for PD1222 genes were cited from the KEGG database.

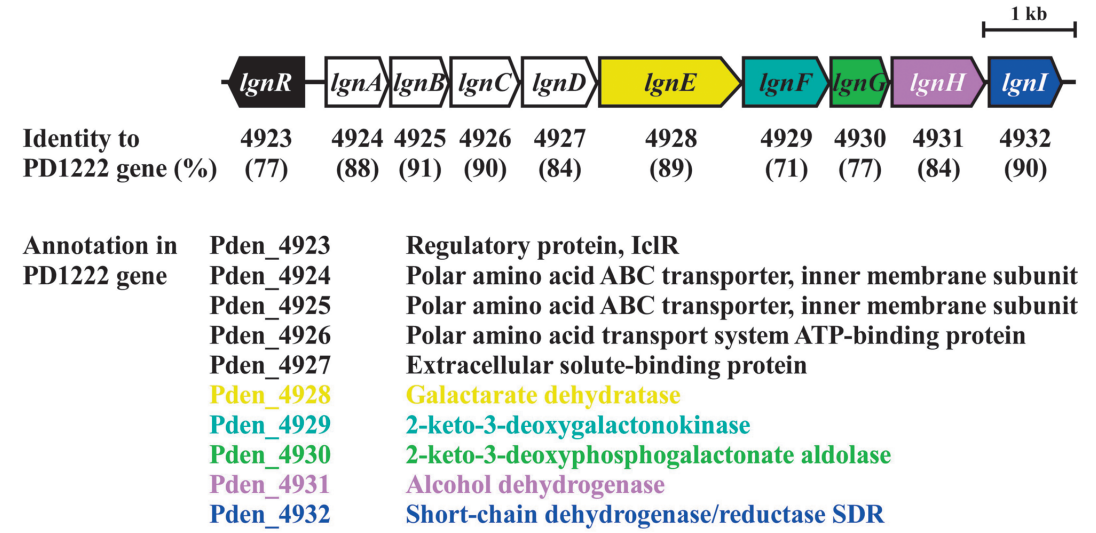

Fig. 2. Gene organization of the *lgn* cluster of strain 43P. The ORFs of the *lgn* cluster are shown with boxes, and the amino acid sequence identity of each ORF with the corresponding gene from strain PD1222 is indicated. Annotations for PD1222 genes were cited from the KEGG database.

strain 43P. Using the N-terminal sequence and an internal amino acid sequence of Pden_4931, two degenerate primers were synthesized and used for cloning of the L-GnDH gene by PCR-based method. We also analyzed the sequence of the region around this gene. We found that the L-GnDH gene was located in a cluster of nine genes, and found an ORF of a putative transcriptional regulator for this cluster in the upstream region. These ORFs showed again high identities of 71–91% in the amino acid sequences with the respective ORFs in strain PD1222 (Pden_4923-Pden_4932). Also, these ORFs showed certain similarities to the genes related to aldonate metabolism. Therefore we assumed that the ORFs in this cluster might be related to a metabolic pathway downstream of L-gluconate, and designated *lgnR* and *lgnA-I* (Fig. 2). Of these ORFs, *lgnE-I* were expected to encode enzymes for L-gluconate metabolism, therefore we analyzed the products of these ORFs further.

Analysis of a metabolic pathway downstream of L-gluconate.

To investigate whether LgnE-I proteins could function as enzymes for L-gluconate metabolism, we overproduced the proteins in *E. coli* using the pET expression system and purified the recombinant proteins. The purified proteins were examined for their enzymatic activity as follows.

LgnH (L-GnDH)

As expected, LgnH showed the same enzymatic activity as the purified L-GnDH. Of the related compounds tested, L-galactonate, the C4 epimer of L-gluconate, could serve as a substrate, but L-mannonate and D-idonate, the C2 and C5 epimer of L-gluconate, respectively, could not. The reaction product from L-gluconate was identified as 5-keto-L-gluconate by HPLC analysis with 5-keto-D-gluconate as a standard.

LgnI (D-idonate dehydrogenase/5-keto-L-gluconate reductase)

LgnI showed an $NADP^+$-dependent dehydrogenase activity against commercial D-idonate, and at the same time, the

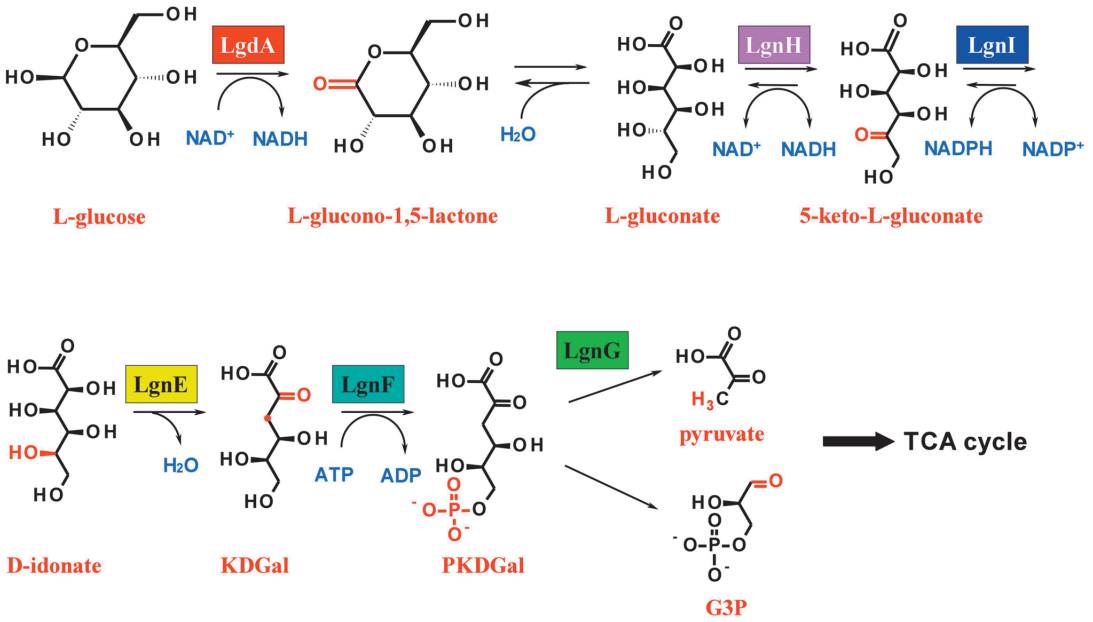

Fig. 3. Model of L-glucose metabolic pathway in strain 43P. The compounds and the enzymes in this pathway are shown, and the atoms and bonds altered by each enzyme reaction are indicated by red color.

enzyme showed a reductase activity against the LgnH reaction product, 5-keto-L-gluconate, in an NADPH-dependent manner. As this enzyme was more active in the reductase reaction than in the dehydrogenase reaction, it is thought to function as the reductase under the physiological conditions. Therefore we concluded that, with LgnH, LgnI catalyses D/L conversion to produce D-idonate from L-gluconate through the dehydrogenase/reductase reactions.

LgnE (D-idonate dehydratase)

LgnE catalyzed conversion of D-idonate to a compound with reducing activity in the presence of Fe^{2+} and DTT, as detected by TLC. The purified reaction product was identified by HPLC and NMR as 2-keto-3-deoxy-D-galactonate (KDGal). Thus we concluded that LgnE was a D-idonate dehydratase.

LgnF (KDGal kinase) and LgnG (6-phospho-KDGal aldolase)

The orthologues of LgnF and LgnG in strain PD1222, the gene products of Pden_4929 and Pden_4930, were annotated as a KDGal kinase and a 6-phospho-KDGal (PKDGal) aldolase, respectively. Therefore we examined LgnF and LgnG proteins for the respective enzymatic activity.

LgnF catalyzed the conversion of KDGal to another compound in the presence of ATP, as determined by TLC analysis. Moreover, addition of LgnG to the reaction mixture resulted in the generation of pyruvate and D-glyceraldehyde-3-phosphate (G3P), which were detected using a commercially available lactate dehydrogenase and G3P dehydrogenase, respectively. Therefore we concluded that LgnF functions as a kinase that phosphorylates KDGal at C6 position, and that LgnG functions as an aldolase that produces pyruvate and G3P from PKDGal.

Conclusion

A model for metabolic pathway of L-glucose in strain 43P is shown in Fig. 3. In general, the metabolic pathways converting aldose sugars to aldonates by oxidation at C1 positions and subsequently converting them to TCA substrates by dehydratase and aldolase reactions are called the Entner-Doudoroff (ED) pathways. Of the ED pathways, the pathway identified here is classified in the semi-phosphorylative (semi-P) ED pathway, because phosphorylation of the substrate occurred after the dehydratase reaction. The semi-P ED pathway is found only in the D-glucose metabolism of some hyperthermophilic archaea[3]. Compared with the pathways found in those archeal strains, the pathway identified in this study is distinct in the reaction steps of the D/L conversion by LgnH/LgnI. Also, the reaction steps after L-gluconate formation in this pathway are similar to those of the L-galactonate metabolic pathway in *E. coli*[4], but the corresponding enzymes does not show any sequence similarities with each other. Therefore we concluded that, together with the uniqueness of the starting substrate, L-glucose, the newly identified pathway differs in many points from the known metabolic pathways.

References

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