PATHWAY OF GALACTITOL CATABOLISM IN *KLEBSIELLA PNEUMONIAE*

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SUMMARY: Cell extracts of galactitol-grown *Klebsiella pneumoniae* phosphorylate galactitol by means of a phosphoenolpyruvate:galactitol phosphotransferase system. Both the product and authentic L-galactitol-1-P are oxidized with NAD^+ by a dehydrogenase to yield D-tagatose-6-P, which is phosphorylated with ATP by a kinase to form D-tagatose-1,6-P_2. This ketohexose diphosphate is cleaved by an aldolase to yield dihydroxyacetone-P and D-glyceraldehyde-3-P. Mutants deficient in either the dehydrogenase, kinase, or aldolase failed to grow on galactitol, indicating that the described pathway is of physiological significance in this organism.

INTRODUCTION

Although hexitol dehydrogenases active on galactitol have been found in various organisms (1-3), the pathway by which galactitol is metabolized in an organism that can use it as a sole source of carbon and energy has not been previously described. Wolff and Kaplan (4) demonstrated that extracts of galactitol-grown *Escherichia coli* can reduce D-tagatose-6-P, presumably to L-galactitol-1-P, but did not demonstrate the phosphorylation of galactitol or the further metabolism of D-tagatose-6-P. Concurrent with our investigation, Lengeler (5) has reported that *E. coli* can transport galactitol via a P-enolpyruvate:hexitol phosphotransferase system, but provided no data on the further metabolism of the phosphorylated hexitol. The present communication provides evidence that the bacterium *Klebsiella pneumoniae* catabolizes galactitol through the pathway: galactitol → L-galactitol-1-P → D-tagatose-6-P → D-tagatose-1,6-P_2 → dihydroxyacetone-P + D-glyceraldehyde-3-P.

MATERIALS AND METHODS

The bacterial strain used was *Klebsiella pneumonia* PRL-R3 (formerly

designated *Aerobacter aerogenes* PRL-R3). The parental strain for mutagenesis was PRL-R3 (U^-), a uracil-requiring auxotroph (6). Mutants VP-8 and VP-14 (galactitol negative) were isolated by methods described previously for strain 012 (6), except that penicillin selection was accomplished with galactitol instead of D-fructose, and the cells were screened on agar plates containing 0.5% (w/v) galactitol and 0.005% (w/v) D-glucose. These two mutants had the wild-type growth pattern on D-glucose, D-mannose, D-fructose, D-galactose, L-arabinose, D-glucitol, and D-mannitol. Mutant A9-1 (D-fructose-6-P kinaseless) was isolated and described previously (6) and was subsequently found to be galactitol negative. Strains VP-8R, VP-14R, and A9-1R were spontaneous revertants of strains VP-8, VP-14, and A9-1, respectively; they were obtained by plating large numbers of mutants on mineral agar plates containing galactitol and selecting large colonies after 48 hours. The growth of cells and preparation of extracts were as previously described (6), except that the carbon source in the mineral medium was 0.5% (w/v) galactitol. For enzyme assays, mutant strains unable to grow on galactitol were grown initially in nutrient broth; then exponential-phase cultures were diluted with an equal volume of nutrient broth containing 1% (w/v) galactitol and were incubated on a shaker at 30° for 6 hours. This time period was found to be optimal for induction of L-galactitol-1-P dehydrogenase and D-tagatose-1,6-P_2 aldolase in the parental strain.

Assays involving pyridine nucleotide coenzymes were measured at 340 nm with a Gilford absorbance-recording spectrophotometer thermostated at 30°. The standard L-galactitol-1-P dehydrogenase assay (0.15 ml) contained 10 μmoles of Tris buffer (pH 8.5), 1.0 μmole of NAD^+, and 0.45 μmole of L-galactitol-1-P. The assay for the reverse reaction (0.15 ml) contained 10 μmoles of Tris buffer (pH 7.75), 0.05 μmole of NADH, and 0.1 μmole of D-tagatose-6-P. Controls were minus the sugar phosphate. D-Tagatose-6-P kinase assays (0.15 ml) contained 10 μmoles of glycylglycine buffer (pH 8.0), 0.05 μmole of ADP, 0.2 μmole of D-tagatose-6-P, 5 μmoles of KCl, 0.03 μmole of $CoCl_2$, and non-rate-limiting amounts of triosephosphate isomerase (EC 5.3.1.1), α-glycerolphosphate dehydrogenase (EC 1.1.99.5), and purified D-tagatose-1,6-P_2 aldolase. Controls were minus ATP. The assay for D-fructose-6-P kinase (EC 2.7.1.11) was previously described (6). The assay for D-tagatose-1,6-P_2 aldolase, which involved production of D-tagatose-1,6-P_2 from D-tagatose-6-P in the assay cuvette, was as previously described (7), except that 5 μmoles of KCl and 0.03 μmole of $CoCl_2$ were also added. Controls were minus D-tagatose-6-P. For all of the above enzymes, one unit of activity is the amount of enzyme that converts 1 μmole of substrate to product per minute. In all cases, the rates were proportional to the amount of the enzyme being assayed.

Protein assays were by the method of Lowry *et al.* (8), or by absorbance at 210 nm (9) using bovine serum albumin as a standard. Purification procedures for enzyme I, enzyme II, and HPr of the phosphotransferase system were described previously (10). L-Galactitol-1-P dehydrogenase was purified 8-fold with 81% recovery by precipitation in 35-70% saturated ammonium sulfate and chromatography on DEAE-cellulose (0 to 0.5 M KCl gradient). D-Tagatose-1,6-P_2 aldolase was purified 48-fold with 51% recovery by chromatography on DEAE-cellulose (0 to 0.45 M KCl gradient), followed by chromatography on Sephadex G-150, and chromatography on DEAE-cellulose (0 to 0.4 M KCl gradient). D-Tagatose-6-P was synthesized as described previously (7), and L-galactitol-1-P was synthesized by reduction of D-galactose-6-P with $NaBH_4$ (11).

RESULTS

In initial experiments with cell extracts from galactitol-grown *K.*

pneumoniae PRL-R3, we were unable to detect the presence of the following activities: galactitol-dependent conversion of ATP to ADP (galactitol kinase); galactitol-dependent reduction of NAD^+ or $NADP^+$ (galactitol dehydrogenase); or epimerization of galactitol, as detected by gas-liquid chromatography (galactitol epimerase). Cell extracts did contain P-enolpyruvate:galactitol phosphotransferase system activity, however. This was most readily demonstrated by reconstituting purified phosphotransferase components (Table I). The activity was dependent upon enzyme I, enzyme II and HPr, and ATP could not substitute for P-enolpyruvate.

TABLE I. Phosphorylation of Galactitol by Reconstituted P-Enolpyruvate: galactitol Phosphotransferase System Components from Galactitol-grown *K. pneumoniae* PRL-R3

Assay	Galactitol Phosphate Formed (nmoles/45 min)
Complete[a]	20.9
Complete minus enzyme I	0.00
Complete minus enzyme II	0.00
Complete minus HPr	0.00
Complete minus galactitol	0.00
Complete minus P-enolpyruvate	0.00
Complete minus P-enolpyruvate plus 1.0 µmole ATP	0.00

[a]The complete reaction mixture (0.2 ml) contained 8.0 µmoles of Tris buffer (pH 7.5), 0.56 µmole of 2-mercaptoethanol, 20 µmoles of galactitol, 0.02 µmole of $MgCl_2$, 1.0 µmole of P-enolpyruvate, 3.0 µmoles of NaF, 11 µg of enzyme II-containing membranes, 2.3 µg of enzyme I (EC 2.7.3.9), and 97 µg of HPr. After 45 min at 30°, the reaction was terminated by heating at 100° for 7 min, and denatured protein was removed by centrifugation. The amount of galactitol phosphate was then measured with the L-galactitol-1-P dehydrogenase assay (see text) containing excess L-galactitol-1-P dehydrogenase.

TABLE II. Enzyme Levels in Parental, Mutant, and Revertant Strains

Strain	Growth on Galactitol[α]	Units/mg Protein			
		L-Galactitol-1-P Dehydrogenase	D-Fructose-6-P Kinase	D-Tagatose-6-P Kinase	D-Tagatose-1,6-P_2 Aldolase
PRL-R3 U^-	+	0.101	0.158	0.095	0.217
A9-1	-	0.021	<0.003	<0.003	0.108
A9-1R	+	0.057	0.127	0.076	0.093
VP-8	-	0.019	0.136	0.089	<0.001
VP-8R	+	0.076	0.167	0.103	0.262
VP-14	-	<0.001	0.109	0.069	0.038
VP-14R	+	0.083	0.152	0.100	0.142

[α] + = wild-type growth in 24 hr; - = no growth in 48 hr.

Extracts from galactitol-induced cells of PRL-R3 U^- also contained L-galactitol-1-P dehydrogenase, D-tagatose-6-P kinase, and D-tagatose-1,6-P_2 aldolase activities (Table II). The dehydrogenase could also be measured in the reverse reaction (i.e., reduction of D-tagatose-6-P with NADH) both with cell extracts (0.037 unit/mg protein) and with the partially purified L-galactitol-1-P dehydrogenase. When partially purified aldolase was allowed to cleave a limiting amount of D-tagatose-1,6-P_2 in the otherwise standard assay, two equivalents of NADH were oxidized, but only one equivalent was oxidized if triose-P isomerase were omitted, indicating that the reaction products are equimolar amounts of dihydroxyacetone-P and D-glyceraldehyde-3-P.

Enzyme levels in the galactitol-negative mutants and revertants are also shown in Table II. Mutant VP-8 was missing D-tagatose-1,6-P_2 aldolase, VP-14 was missing L-galactitol-1-P dehydrogenase, and A9-1, which was previously shown to be missing D-fructose-6-P kinase (6), was also missing D-tagatose-6-P kinase activity. Revertants (VP-8R, VP-14R, and A9-1R) regained the missing enzyme activities.

DISCUSSION

These data provide evidence for the presence of four enzyme activities in cell extracts of galactitol-grown *K. pneumoniae* that are instrumental in galactitol catabolism. These activities are a P-enolpyruvate:galactitol phosphotransferase system, L-galactitol-1-P dehydrogenase, D-tagatose-6-P kinase, and D-tagatose-1,6-P_2 aldolase. The phosphotransferase system required the addition of all three protein components for production of the galactitol phosphate product, and ATP could not substitute for P-enolpyruvate. Since the galactitol phosphate product of the phosphotransferase served as substrate for the partially purified L-galactitol-1-P dehydrogenase, it seems probable that its identity is L-galactitol-1-P. The inability to detect an ATP:galactitol phosphotransferase, a galactitol dehydrogenase, or a galactitol epimerase in extracts is consistent with the P-enolpyruvate:

galactitol phosphotransferase system having a primary function in galactitol catabolism.

Oxidation of L-galactitol-1-P and reduction of D-tagatose-6-P were observed both with cell extracts and with partially purified L-galactitol-1-P dehydrogenase. Further catabolism of D-tagatose-6-P involved D-tagatose-6-P kinase and D-tagatose-1,6-P_2 aldolase. D-Tagatose-6-P was phosphorylated by the kinase, forming a product that was cleaved by D-tagatose-1,6-P_2 aldolase to yield triosephosphates. The aldolase, in assays coupled with triose-P isomerase and α-glycerol-P dehydrogenase, cleaved enzymatically synthesized D-tagatose-1,6-P_2 to dihydroxyacetone-P and D-glyceraldehyde-3-P, the expected products.

D-Tagatose-6-P kinase and D-tagatose-1,6-P_2 aldolase were previously described as components of the D-tagatose-6-P pathway of lactose and D-galactose catabolism in *Staphylococcus aureus* (7, 12) and in Group N streptococci (13). In contrast, *K. pneumoniae (A. aerogenes)* metabolizes D-galactose through the Leloir pathway (13).

Mutant strains missing either L-galactitol-1-P dehydrogenase (VP-14), D-tagatose-6-P kinase (A9-1), or D-tagatose-1,6-P_2 aldolase (VP-8) were unable to grow on galactitol, whereas revertants that regained their ability to grow on galactitol also regained the missing activities. These data confirm the participation of these enzymes in galactitol catabolism and establish that this is the sole physiological pathway for galactitol catabolism in this organism. Mutant A9-1 had originally been isolated as a D-fructose-6-P kinase-deficient strain (6). Our current findings that D-tagatose-6-P kinase is also absent in this mutant, and that both activities are regained in the revertant, suggest that these two kinase activities are manifestations of the same protein. Further evidence for this dual specificity, as well as further characterization of the kinase, L-galactitol-1-P dehydrogenase, and D-tagatose-1,6-P_2 aldolase will be presented elsewhere.

In summary, the data in this report provide evidence for the catabolism

of galactitol in *K. pneumoniae* by the pathway: galactitol → L-galactitol-1-P → D-tagatose-6-P → D-tagatose-1,6-P_2 → dihydroxyacetone-P + D-glyceraldehyde-3-P. This is the first report of a catabolic pathway for galactitol in any organism.

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REFERENCES

1. Shaw, D.R.D. (1956) *Biochem. J.* 64, 394-405.
2. Weimberg, R. (1962) *Biochem. Biophys. Res. Commun.* 8, 442-445.
3. Barnett, J.A. (1968) *J. Gen. Microbiol.* 52, 131-159.
4. Wolff, J.B., and Kaplan, N.O. (1956) *J. Bacteriol.* 71, 557-564.
5. Lengeler, J. (1975) *J. Bacteriol.* 124, 26-38, 39-47.
6. Sapico, V., Hanson, T.E., Walter, R.W., and Anderson, R.L. (1968) *J. Bacteriol.* 96, 51-54.
7. Bissett, D.L., and Anderson, R.L. (1973) *Biochem. Biophys. Res. Commun.* 52, 641-647.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275.
9. Tombs, M.P., Souter, F., and MacLagen, N.F. (1959) *Biochem. J.* 73, 167-171.
10. Walter, R.W., and Anderson, R.L. (1973) *Biochem. Biophys. Res. Commun.* 52, 93-97.
11. Wolff, J.B., and Kaplan, N.O. (1956) *J. Biol. Chem.* 218, 849-869.
12. Bissett, D.L., and Anderson, R.L. (1974) *J. Bacteriol.* 119, 698-704.
13. Bissett, D.L., and Anderson, R.L. (1974) *J. Bacteriol.* 117, 318-320.