D-*GALACTONATE UTILISATION BY ENTERIC BACTERIA

The catabolic pathway in *Escherichia coli*

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1. Introduction

The absence of a reducing group in the aldonic sugar acids effectively prevents them from participating in polysaccharide formation and they occur mainly as metabolic intermediates in the catabolism of other sugars. In *Escherichia coli* gluconate 6-phosphate is an intermediate in the oxidative catabolism of glucose [1], and mannonate and altronate are intermediates in the catabolism of glucuronate and galacturonate, respectively [2]. Although galactonate is known as an intermediate in galactose metabolism in Pseudomonads [3] its utilisation by enteric bacteria does not seem to have been studied (see [4] for *Salmonella typhimurium* LT-2).

We have observed that a variety of enteric bacteria grow readily on galactonate as sole carbon and energy source and this paper describes the pathway for galactonate catabolism in *E. coli.*

2. Materials and methods

The bacteria used during the course of this work were the *E. coli* strains: NCTC 9001 (neotype), B, Crookes and the K-12 derivatives K10, PA309 [5], X289 [6], DF 1070 *gnd edd* [7] and A1201 *kga* [8]; *S. typhimurium* LT-2; and *Pseudomonas saccharophila.* The gluconate-negative mutants DF 1070 and A1201 were kindly provided by Dr P. Faik and the other strains were from laboratory stocks.

The enteric bacteria were grown in appropriately supplemented minimal medium 63 [9] with either galactonate, glucose, gluconate, galactose (all at 10 mM) or glycerol (20 mM) as carbon source. *P. saccharophila* was grown as previously described [10]. The liquid media were solidified as appropriate by the incorporation of 1.6% Oxoid bacteriological agar.

For the preparation of cell-free extracts bacteria from 100 ml medium were harvested in the late logarithmic phase of growth, suspended in 4 ml 50 mM imidazole–HCl/0.5 mM dithiothreitol (DTT)/ 0.6 mM $FeSO_4$ buffer, pH 7.0 and disrupted by exposure to ultrasonic oscillations in an MSE 100-W ultrasonic disintegrator (operating at 8 μm peak-to-peak amplitude) at 0°C for 1 min. The suspensions were centrifuged at 25 000 × *g* for 15 min at 4°C to remove cell debris.

For assays involving measurement of $NADH_2$ oxidation the supernatant solutions were further centrifuged at 150 000 × *g* for 1.5 h at 4°C to remove the particulate $NADH_2$ oxidase activity. Soluble protein was measured by the biuret method [11] using crystalline bovine serum albumin as the standard.

Ammonium sulphate fractionation was carried out at 0°C. Solid ammonium sulphate (22.6 g/100 ml) was added to give 40% saturation and after stirring for 30 min the precipitate was removed by centrifugation. More ammonium sulphate (5.8 g/100 ml) was added to the supernatant to give 50% saturation and after stirring for 30 min the precipitate was collected

* The sugars are all of the D-series unless shown otherwise

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by centrifugation and dissolved in an amount of 50 mM imidazole–HCl/0.5 mM DTT/0.6 mM $FeSO_4$ buffer, pH 7.0 equivalent to one-tenth of the volume of the original extract.

For gel-filtration the sample was applied to a 45 × 2.5 cm column of Sephadex G-150 equilibrated with 50 mM imidazole–HCl/0.5 mM DTT buffer, pH 7.0. The column was eluted at 4°C with the same buffer and 2 ml fractions were collected.

Pyruvate formation was measured at 30°C in a reaction mixture that contained in 1 ml: 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris)–HCl buffer, pH 7.5 (100 μmol), $MgCl_2$ (5 μmol), $NADH_2$ (0.15 μmol), ATP (2 μmol), crystalline lactate dehydrogenase (5 μg), sodium galactonate (5 μmol) or sodium 2-oxo-3-deoxygalactonate (0.5 μmol) and bacterial extract.

After measurement of the endogenous rate of $NADH_2$ oxidation the reaction was initiated by addition of the galactonate or 2-oxo-3-deoxygalactonate. Glyceraldehyde 3-phosphate formation was measured in an identical assay but with crystalline triosephosphate isomerase/α-glycerophosphate dehydrogenase (10 μg) instead of lactate dehydrogenase.

Galactonate dehydratase (EC 4.2.1.6) was measured at 30°C in a reaction mixture that contained in 5 ml: Tris–HCl buffer, pH 7.5 (500 μmol), $MgCl_2$ (25 μmol), galactonate (25 μmol) and bacterial extract. Samples (1 ml) were removed at various times, heated at 100°C for 2 min then centrifuged to remove any precipitate. The 2-oxo-3-deoxygalactonate in the supernatants was measured with the semicarbazide reagent [12] in final vol. 2 ml. Under these conditions 1 μmol 2-oxo-3-deoxygalactonate has an absorbance of 5.7.

The assay for 2-oxo-3-deoxygalactonate kinase (EC 2.7.1.58) at 30°C contained in 1 ml: Tris–HCl buffer, pH 7.5 (100 μmol), $MgCl_2$ (5 μmol), ATP (2 μmol), $NADH_2$ (0.15 μmol), PEP (1 μmol), crystalline lactate dehydrogenase (5 μg), crystalline pyruvate kinase (2 μg), 2-oxo-3-deoxygalactonate (0.5 μmol) and bacterial extract. After measurement of the endogenous rate due to $NADH_2$ oxidase and adenosine triphosphatase (EC 3.6.1.3) the reaction was started by the addition of the 2-oxo-3-deoxygalactonate.

The assay for 2-oxo-3-deoxy-6-phosphogalactonate aldolase (EC 4.1.2.21) at 30°C contained in 1 ml: Tris–HCl buffer, pH 7.5 (100 μmol), $MgCl_2$ (5 μmol), ATP (2 μmol), $NADH_2$ (0.15 μmol), 2-oxo-3-deoxygalactonate (0.5 μmol), partially purified 2-oxo-3-deoxygalactonate kinase (0.03 unit), crystalline lactate dehydrogenase (5 μg) and bacterial extract. The mixture was incubated for 10 min in the absence of bacterial extract to permit 2-oxo-3-deoxy-6-phosphogalactonate to be produced and then the endogenous rate of $NADH_2$ oxidation was measured.

The reaction was started by the addition of bacterial extract. The bacterial $NADH_2$ oxidase was measured in a separate assay. Solutions of 2-oxo-3-deoxygalactonate were assayed by the 2-thiobarbituric acid method [13] and the factor for 2-oxo-3-deoxygluconate [14] was assumed to apply to 2-oxo-3-deoxygalactonate. nate.

Biochemicals and crystalline enzymes were purchased from Boehringer (London) Ltd. Galactonolactone was obtained from Sigma London Ltd, and converted to sodium galactonate by titration with NaOH.

3. Results and discussion

Various *E. coli* K-12 derivatives, strain B, and the neotype strain NCTC 9001, as well as *S. typhimurium* LT-2 grew readily on galactonate as sole carbon and energy source. In liquid medium the doubling time for growth of *E. coli* K10 on either glucose, gluconate or galactonate at 37°C was almost identical at 65–70 min.

The ability of *E. coli* to utilise galactonate as readily as gluconate, coupled with the similarities in the catabolic pathways for the pairs of sugar acids glucuronate and galacturonate [2] on the one hand and glucarate and galactarate [15] on the other, suggested that the pathway for galactonate catabolism might be similar to that already established for gluconate catabolism [16,17]. To test this possibility *E. coli* K-12 mutants that were unable to grow on gluconate, strain DF 1070 deficient in 6-phosphogluconate dehydratase (EC 4.2.1.12) and strain A1201 deficient in 2-oxo-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14), were tested for their ability to grow on galactonate. Both mutants grew normally, suggesting that neither of these enzymes was involved in galactonate catabolism.

Nevertheless, there were similarities between gluconate and galactonate catabolism. When crude extracts prepared from galactonate-grown *E. coli* strain K10 were incubated with galactonate, pyruvate was produced but only if ATP was also included. Thus it was possible that galactonate catabolism involved a series of reactions that paralleled those for gluconate catabolism but involved at least some distinct enzymes. Alternatively, galactonate catabolism might be similar to mannonate and altronate catabolism [2] with dehydration of the non-phosphorylated sugar acid producing a 2-oxo-3-deoxy derivative that was then phosphorylated and cleaved to produce pyruvate and glyceraldehyde 3-phosphate (fig.1).

To facilitate investigation of the catabolic pathway crude extracts prepared from galactonate-grown cells were fractionated with ammonium sulphate and the fraction precipitating between 40–50% saturation was found to catalyse a rapid formation of pyruvate from galactonate plus ATP. In such assays the rate of pyruvate formation was increased almost 3-fold if the incubation was allowed to proceed for 20 min before addition of the ATP. This suggested that the galactonate could be transformed in the absence of ATP, and a substance that was produced gave the characteristic spectrum of a 2-oxo acid with semicarbazide [12] and *O*-phenylenediamine [18]. When assayed by the highly sensitive method for 2-oxo-3-deoxy sugar acids [13] that involves periodate oxidation followed by reaction with 2-thiobarbituric acid it gave a characteristic spectrum with a maximum at 548-550 nm. It failed to react with 2,4-dinitrophenylhydrazine, a property reported earlier for 2-oxo-3-deoxygalactonate [3]. Thus it seems likely that the

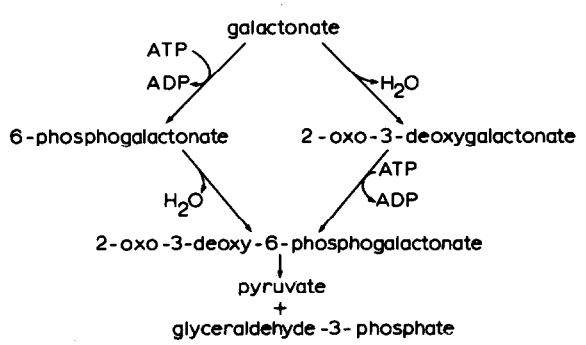

Fig.1. Possible pathways for the catabolism of galactonate.

oxo-compound formed from galactonate is 2-oxo-3-deoxy-galactonate, a substance known to be an intermediate in galactose catabolism in *P. saccharophila* [3]. This suggestion was supported by the finding that crude extracts prepared from galactose-grown *P. saccharophila* catalysed pyruvate formation from the oxo-compound in the presence of ATP whereas extracts prepared from glucose-grown *P. saccharophila* were inactive. Similarly 2-oxo-3-deoxy-galactonate prepared as described by DeLey and Doudoroff [3] was readily converted into pyruvate by cell-free extracts prepared from galactonate-grown *E. coli* but not by extracts prepared from glucose-, gluconate-, or glycerol-grown *E. coli.*

When 2-oxo-3-deoxygalactonate and ATP were incubated with crude extracts or 40–50% ammonium sulphate fraction prepared from galactonate-grown cells, ADP was produced, indicating the presence of an ATP-dependent 2-oxo-3-deoxygalactonate kinase. That the other product of this reaction was 2-oxo-3-deoxy-6-phosphogalactonate was suggested by its conversion to equimolar amounts of pyruvate and glyceraldehyde 3-phosphate (see later). The kinase was partially purified by gel-filtration of the 40–50% ammonium sulphate fraction on a Sephadex G-150 column. It was eluted after the galactonate dehydratase but measurement of pyruvate formation from 2-oxo-3-deoxygalactonate plus ATP indicated that the kinase and a putative 2-oxo-3-deoxy-6-phosphogalactonate aldolase were eluted together. However, the aldolase activity in the fractions was unstable and after four days at 4°C virtually all the aldolase activity was lost. The kinase was stable under these conditions and the enzyme from pooled column fractions was precipitated with 80% saturated ammonium sulphate and dissolved in a small volume of 50 mM imidazole–HCl/0.5 mM DTT/0.6 mM $FeSO_4$ buffer, pH 7.0. This concentrated 2-oxo-3-deoxygalactonate kinase was used to phosphorylate 2-oxo-3-deoxygalactonate in a 1 ml reaction mixture (see Materials and methods) to produce the substrate for the 2-oxo-3-deoxy-6-phosphogalactonate aldolase.

When the amount of 2-oxo-3-deoxygalactonate was made the limiting component in such kinase and aldolase assays equivalent amounts of ADP, pyruvate, and glyceraldehyde 3-phosphate were produced, as shown in table 1. Thus it seems that galactonate catabolism in *E. coli* is similar to that reported for

Table 1
The stoichiometry of ADP, pyruvate and glyceraldehyde 3-phosphate formation from 2-oxo-3-deoxygalactonate + ATP

Compound	Amount added or produced (μmol)
2-Oxo-3-deoxygalactonate	0.087
ADP	0.089
Pyruvate	0.088
Glyceraldehyde 3-phosphate	0.088

The compounds were estimated as described in the Materials and methods section

P. saccharophila [3] and distinct from the catabolism of gluconate.

It is proposed that three enzymes, galactonate dehydratase, 2-oxo-3-deoxygalactonate kinase, and 2-oxo-3-deoxy-6-phosphogalactonate aldolase are responsible for the unique reactions involved in the conversion of internal galactonate into intermediates of the glycolytic pathway. These three enzymes were present at high activity in extracts prepared from galactonate-grown cells (table 2) but could not be detected in extracts prepared from glycerol-, gluconate-, or galactose-grown cells.

Further evidence in support of the proposed pathway was obtained by investigation of *E. coli* Crookes strain. This strain failed to grow on galactonate plates but after 2–3 days incubation at 37°C galactonate positive clones appeared. Both the original strain and a galactonate-positive mutant were grown on glycerol plus galactonate and cell-free extracts prepared and assayed for the galactonate catabolic enzymes. Whereas none of the enzymes could be detected in the original strain, all three were present at high activity in the galactonate-positive mutant (table 2).

Since galactonate does not seem to be widespread in nature the ready growth on this sugar acid raises the possibility that *E. coli* may have the genetic potential for a galactose degradative pathway of the kind found in *P. saccharophila* [3]. In this context the recent finding of a galactose dehydrogenase in an *E. coli* mutant [19] is most interesting, although the product of this enzyme was tentatively identified as 3-oxo-galactose. However, the failure to detect the dehydratase, kinase, or aldolase activities in wild-type cells grown on galactose suggests that the galactonate catabolic enzymes do not normally participate in galactose catabolism in *E. coli*. Their role would seem to be the catabolism of exogenous galactonate.

Table 2
Activities of the galactonate catabolic enzymes

	Specific activities (μmol substrate transformed.min^{-1}.mg protein^{-1})		
Organism	Galactonate dehydratase	2-Oxo-3-deoxy galactonate kinase	2-Oxo-3-deoxy 6-phosphogalactonate aldolase
E. coli K10	0.06	0.24	0.30
S. typhimurium LT-2	0.05	0.06	0.21
E. coli Crookes (galactonate$^+$-mutant)	0.05	0.18	0.29

E. coli K10 and *S. typhimurium* LT-2 were grown on galactonate. *E. coli* Crookes galactonate$^+$-mutant was grown on glycerol + galactonate

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