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Directed Evolution of a Second Xylitol Catabolic Pathway in *Klebsiella pneumoniae*

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Klebsiella pneumoniae **PRL-R3 has inducible catabolic pathways for the degradation of ribitol and D-arabitol but cannot utilize xylitol as a growth substrate. A mutation in the *rbtB* regulatory gene of the ribitol operon permits the constitutive synthesis of the ribitol catabolic enzymes and allows growth on xylitol. The evolved xylitol catabolic pathway consists of an induced D-arabitol permease system that also transports xylitol, a constitutively synthesized ribitol dehydrogenase that oxidizes xylitol at the C-2 position to produce D-xylulose, and an induced D-xylulokinase from either the D-arabitol or D-xylose catabolic pathway. To investigate the potential of *K. pneumoniae* to evolve a different xylitol catabolic pathway, strains were constructed which were unable to synthesize ribitol dehydrogenase or either type of D-xylulokinase but constitutively synthesized the D-arabitol permease system. These strains had an inducible L-xylulokinase; therefore, the evolution of an enzyme which oxidized xylitol at the C-4 position to L-xylulose would establish a new xylitol catabolic pathway. Four independent xylitol-utilizing mutants were isolated, each of which had evolved a xylitol-4-dehydrogenase activity. The four dehydrogenases appeared to be identical because they comigrated during nondenaturing polyacrylamide gel electrophoresis. This novel xylitol dehydrogenase was constitutively synthesized, whereas L-xylulokinase remained inducible. Transductional analysis showed that the evolved dehydrogenase was not an altered ribitol or D-arabitol dehydrogenase and that the evolved dehydrogenase structural gene was not linked to the pentitol gene cluster. This evolved dehydrogenase had the highest activity with xylitol as a substrate, a K_m for xylitol of 1.4 M, and a molecular weight of 43,000.**

The evolution of biochemical pathways for the catabolism of novel substrates has been studied in several bacterial systems (4, 15). *Klebsiella* strains that have mutated to gain the ability to utilize xylitol have been investigated to determine the biochemical and genetic bases of the evolved xylitol pathway (12, 16, 24). *K. pneumoniae* PRL-R3 (previously known as *Aerobacter aerogenes* PRL-R3) has inducible catabolic pathways for the degradation of 5 of the 12 pentoses and pentitols (Fig. 1) (17). The two pentitols, ribitol and D-arabitol, are degraded by similar pathways, with both being oxidized to a 2-ketopentose and then phosphorylated. The ribitol catabolic enzymes, ribitol dehydrogenase (EC 1.1.1.56) and D-ribulokinase (EC 2.7.1.47), are induced by D-ribulose, whereas D-arabitol dehydrogenase (EC 1.1.1.11) and the D-xylulokinase (EC 2.7.1.17) of the D-arabitol pathway are induced by D-arabitol. Pentitol transport systems are also induced during growth on ribitol or D-arabitol (24; J. P. Mays and R. P. Mortlock, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K113, p. 195).

Xylitol-utilizing mutants of *K. pneumoniae* PRL-R3 studied previously have all been shown to possess a xylitol catabolic pathway that is similar in strategy to the ribitol and D-arabitol pathways. Xylitol is oxidized at the C-2 position to produce D-xylulose, which is then phosphorylated by a D-xylulokinase to produce D-xylulose-5-phosphate (Fig. 2) (16). The origins of the dehydrogenase and kinase activities of this evolved xylitol pathway have been determined previously.

The D-arabitol permease system and the D-xylulokinase of the existing D-arabitol pathway are utilized for xylitol degradation (12, 24). Low levels of the D-arabitol catabolic enzymes and permease system are induced during xylitol metabolism. Presumably, some of the D-xylulose produced by the oxidation of xylitol is converted to D-arabitol, the inducer of the D-arabitol operon, by basal levels of D-arabitol dehydrogenase. The D-arabitol permease system has been shown to transport xylitol, and the D-xylulokinase of the D-arabitol pathway can phosphorylate the D-xylulose produced. The xylitol dehydrogenase activity in xylitol-utilizing mutants is due to ribitol dehydrogenase. Ribitol dehydrogenase has low catalytic activity for the oxidation of xylitol at the C-2 position, producing D-xylulose. However, the inducer of the ribitol operon, D-ribulose, is not formed during xylitol degradation. The mutation establishing the xylitol catabolic pathway is in the *rbtB* regulatory gene and permits the constitutive synthesis of the ribitol catabolic enzymes. The constitutively synthesized ribitol dehydrogenase oxidizes xylitol to D-xylulose, resulting in the induction of the D-arabitol catabolic enzymes and growth on xylitol (12, 16). *Escherichia coli* C strains, which also possess ribitol and D-arabitol pathways, mutate to utilize xylitol by the same mechanism, the constitutive synthesis of ribitol dehydrogenase (23).

A general finding of studies in experimental metabolic evolution is that the repetition of selection experiments produces the same types of mutations in the same genes and in the same order. This can be interpreted to mean that the evolutionary choices provided by a genetic background are very limited (4, 8–10, 15). Since all xylitol-utilizing mutants of *K. pneumoniae* previously studied have employed ribitol dehydrogenase to oxidize xylitol to D-xylulose, we wished to determine whether the genetic background of *K. pneumoniae* PRL-R3 provides the evolutionary potential to evolve other xylitol catabolic pathways. The oxidation of xylitol at the C-4 position rather than at the C-2 position would

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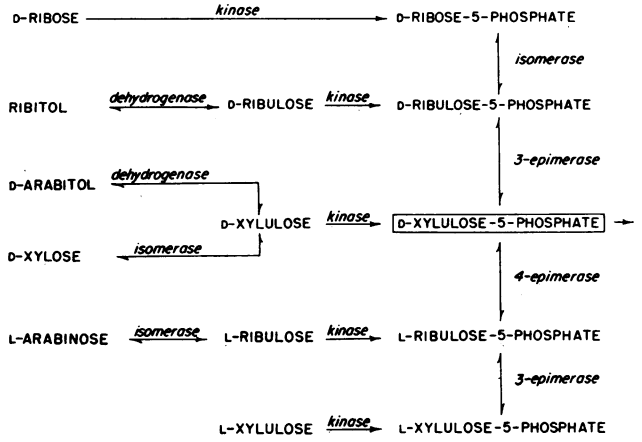

FIG. 1. Inducible pentose and pentitol catabolic pathways in *K. pneumoniae* PRL-R3.

produce L-xylulose (Fig. 2). *K. pneumoniae* PRL-R3 is known to be capable of inducing an L-xylulokinase (EC 2.7.1.53) that phosphorylates L-xylulose to the 5-phosphate and an L-xylulose-5-phosphate 3-epimerase (17). Therefore, the evolution of a xylitol transport system and a xylitol dehydrogenase which oxidized xylitol at the C-4 position should complete a different and novel xylitol catabolic pathway. To investigate the potential of *K. pneumoniae* to evolve this postulated pathway, we constructed strains that were unable to evolve the normal ribitol dehydrogenase-D-xylulokinase xylitol pathway. These strains also constitutively synthesized the D-arabitol permease system, allowing xylitol transport. Selective pressure was now directed to the evolution of a xylitol-4-dehydrogenase activity, which was postulated to be the sole requirement for establishing the new pathway.

This investigation has shown that *K. pneumoniae* PRL-R3 has the potential to evolve this novel xylitol catabolic pathway. A combination of structural and regulatory mutations directed selective pressure to the evolution of a xylitol-4-dehydrogenase and established a second xylitol catabolic pathway in *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *K. pneumoniae* strains used in this investigation are listed in Table 1. All strains were derived from a *K. pneumoniae* PRL-R3 uracil auxotroph. Cells were grown aerobically at 30°C on minimal medium (2) supplemented with 0.5% carbohydrate or 0.5% casein hydrolysate and 0.0025% uracil or all three. Solid medium was solidified with 0.8% Gelrite (Kelco). Growth of cells was monitored by the optical density at 660 nm.

Isolation of mutants. D-Arabinose-utilizing mutants were isolated as previously described (20). Ribitol-negative mutants were isolated after ethyl methanesulfonate (EMS) mutagenesis (14) and enrichment. Penicillin enrichments were done basically by the procedure of Davis (5), with the penicillin concentration increased to 6 mg/ml, cycloserine added at 0.5 mg/ml, and both added only after growth had begun. Xylitol-positive mutants of strains DM704 and DM708 were isolated in the following manner. Approximately 1.1×10^{10} cells of strain DM704, strain DM704 mutagenized with EMS, strain DM708, or strain DM708 mutagenized with EMS were inoculated into 250-ml flasks containing 25 ml of xylitol-uracil-minimal salts medium and incubated aerobically at 30°C. After 12 days, growth was observed in each of the four flasks. Phage P1CMc1r100 was used to isolate P1-sensitive mutants as described elsewhere (7).

Cell extracts. Cells were harvested from stationary-phase cultures by centrifugation at 12,000 × *g* for 10 min at 4°C, washed once in ice-cold 50 mM Tris–50 mM glycine–0.2 mM 2-mercaptoethanol buffer, and suspended in the same buffer. The cell suspension was placed in an ice bath and then sonicated for three 20-s bursts at a 20-W setting with a Braunsonic sonicator. Cell debris was removed by centrifugation at 39,000 × *g* for 15 min at 4°C. Protein was determined by the method of Bradford (1) with reagents obtained from Bio-Rad Laboratories.

Enzyme assays. Ribitol dehydrogenase activity (measured as D-ribulose reductase activity), D-ribulokinase activity, and D-xylulokinase activity were determined as previously described (2). L-Xylulose reductase activity and L-xylulokinase activity determinations were similar to those of D-ribulose reductase and D-ribulokinase except that L-xylulose was used as the substrate at a concentration of 50 mM. Xylitol dehydrogenase activity was measured by monitoring the production of NADH at 340 nm with xylitol as the substrate. Assays were performed in 50 mM xylitol–0.83 mM NAD^+–50 mM Tris–50 mM glycine–0.2 mM 2-mercaptoethanol. Substrate specificity determinations used the same assay procedure with each substrate at a concentration of 50 mM.

Phage production and transductions. Transducing-phage lysates were obtained from P1CMc1r100 lysogens by heat shock (22). Transductions were carried out as outlined by Goldberg et al. (7) with a lysogenic recipient strain and phage added at a multiplicity of infection between 2 and 5.

Electrophoresis and molecular weight determinations. Electrophoresis under nondenaturing conditions was performed by modifying the procedure of Laemmli (11) as follows: sodium dodecyl sulfate was omitted from all buffers, and 2-mercaptoethanol was added to 0.5 mM; gels were photopolymerized with 0.0075% ammonium persulfate–0.00025% riboflavin phosphate–0.2% *N,N,N',N'*-tetramethylethylenediamine; and gels were run at 10 mA for 10 min before the sample was loaded. Gels were stained for protein with

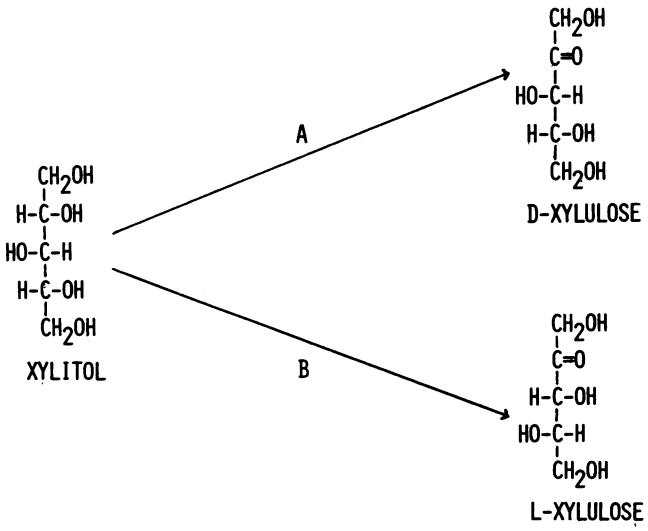

FIG. 2. Oxidation of xylitol at the C-2 (A) and C-4 (B) positions.

TABLE 1. *K. pneumoniae* strains used

Strain	Description[a]	Source
261-5	Xyl$^-$ Ura$^-$	This laboratory
DM275	RDH^c Xyl$^-$ Ura$^-$	Xyt$^+$ of 261-5
DM516	ADH^c DXK$^-$ Xyl$^-$ Ura$^-$	This laboratory
DM5169	ADH^c DXK$^-$ Xyl$^-$ Ura$^-$ P1^s (P1CMclr100)	P1^s of DM516
DM601	ADH^c DXK$^-$ Xyl$^-$ D-Ara$^+$ ura$^-$	D-Ara$^+$ of DM516
DM704	ADH^c DXK$^-$ RDH$^-$ Xyl$^-$ D-Ara$^+$ Ura$^-$	RDH$^-$ (EMS^b) of DM601
DM708	ADH^c DXK$^-$ RDH$^-$ Xyl$^-$ D-Ara$^+$ Ura$^-$	RDH$^-$ (EMS) of DM601
DM750	ADH$^-$ DXK$^-$ RDH$^-$ Xyl$^-$ D-Ara$^+$ Ura$^-$	ADH$^-$ (EMS) of DM708
DM7509	ADH$^-$ DXK$^-$ RDH$^-$ Xyl$^-$ D-Ara$^+$ Ura$^-$ P1^s (P1CMclr100)	P1^s of DM750
DM801	ADH^c DXK$^-$ RDH$^-$ Xyt$^+$ Xyl$^-$ D-Ara$^+$ Ura$^-$	Xyt$^+$ of DM704
DM802	ADH^c DXK$^-$ RDH$^-$ Xyt$^+$ Xyl$^-$ D-Ara$^+$ Ura$^-$	Xyt$^+$ of DM704
DM803	ADH^c DXK$^-$ RDH$^-$ Xyt$^+$ Xyl$^-$ D-Ara$^+$ Ura$^-$	Xyt$^+$ of DM708
DM804	ADH^c DXK$^-$ RDH$^-$ Xyt$^+$ Xyl$^-$ D-Ara$^+$ Ura$^-$	Xyt$^+$ of DM708
DM8039	ADH^c DXK$^-$ RDH$^-$ Xyt$^+$ Xyl$^-$ D-Ara$^+$ Ura$^-$ P1^s (P1CMclr100)	P1^s of DM803
DM8889	ADH$^-$ DXK$^-$ RDH$^-$ Xyt$^+$ Xyl$^-$ D-Ara$^+$ Ura$^-$ P1^s (P1CMclr100)	DM8039 × DM7509

[a] Xyl, D-Xylose; Ura, uracil; Xyt, xylitol; D-Ara, D-arabinose; P1^s, P1 sensitive; ADH, D-arabitol dehydrogenase; DXK, D-xylulokinase; RDH, ribitol dehydrogenase; c, constitutive.
[b] EMS mutagenesis.

Coomassie blue or for dehydrogenase activity by the method of Neuberger et al. (18). The molecular weight of xylitol dehydrogenase was estimated by nondenaturing gel electrophoresis (6). Crude cell extracts were separated in 6, 7, 8, 9, and 10% gels, and the dehydrogenase band was identified after staining for dehydrogenase activity.

Purification of xylitol-4-dehydrogenase. *K. pneumoniae* DM8889 was grown in four 200-ml volumes of xylitol-casein hydrolysate-uracil-minimal salts medium at 30°C to the stationary phase. The cells were harvested by centrifugation at 12,000 × *g* for 15 min, washed once with four 25-ml volumes of 50 mM Tris–50 mM glycine–0.2 mM 2-mercaptoethanol buffer, and suspended in 40 ml of the same buffer. Cells were sonicated as described above with four 20-s bursts. Cell debris was removed by centrifugation at 39,000 × *g*. The following purification procedure was carried out at 4°C. The cell extract was loaded onto a DEAE-cellulose column (3 by 20 cm) previously equilibrated with 10 mM potassium phosphate (pH 7.2)–0.2 mM 2-mercaptoethanol buffer. The column was eluted with a step gradient consisting of 100 ml of each of the following: 50, 70, 80, 90, and 100 mM potassium phosphate (pH 7.2) with 0.2 mM 2-mercaptoethanol buffer. Fractions (7.5 ml) were collected and assayed for xylitol dehydrogenase activity. Active fractions were pooled, and 30 ml of the pooled fractions was concentrated to a volume of 250 μl and dialyzed against 1 liter of 50 mM Tris–50 mM glycine–0.2 mM 2-mercaptoethanol buffer in a vacuum dialysis concentrator (Bio-Molecular Dynamics). The concentrate was loaded onto a preparative electrophoresis unit (Bethesda Research Laboratories). A 4-cm 7% polyacrylamide gel with a 1.5-cm stacking gel, prerun at 10 mA for 10 min, was used in the fractionation. The gel was run at 6 mA, and after the tracking dye front had reached the elution chamber, 1.5-ml fractions were collected at a flow rate of 0.4 ml/min. Fractions were assayed for dehydrogenase activity, and active fractions were pooled. The pooled fractions were concentrated and dialyzed as described above and the sample was stored at −20°C.

Chemicals. Xylitol was a gift from Hoffmann-La Roche, Inc. D-Ribulose was prepared as previously described (19). D-Xylulose was prepared by a similar procedure from D-arabitol with *K. pneumoniae* DM516. L-Xylulose was prepared by a similar procedure from xylitol by *Erwinia uredovora* DM122. Strain DM122 is constitutive for the synthesis of a xylitol-4-dehydrogenase and is unable to synthesize L-xylulokinase. Electrophoresis chemicals were obtained from Bio-Rad Laboratories.

RESULTS

Rationale and construction of xylitol-negative strains. All xylitol-utilizing *Klebsiella* mutants previously examined have gained the ability to degrade xylitol as the result of a mutation permitting the constitutive synthesis of ribitol dehydrogenase. To prevent the evolution of this xylitol pathway during attempts to evolve a xylitol catabolic pathway in which L-xylulose is an intermediate, we wished to construct strains of *K. pneumoniae* that lacked ribitol dehydrogenase and D-xylulokinase activities. *K. pneumoniae* has been shown to possess an inducible L-xylulokinase and an L-xylulose-5-phosphate 3-epimerase (17). A xylitol transport system and a dehydrogenase that oxidizes xylitol at the C-4 position to produce L-xylulose should complete a novel xylitol catabolic pathway. To supply the xylitol transport activity, the strains used in the directed-evolution experiments would have a mutation permitting the constitutive synthesis of the D-arabitol permease system. We predicted that the evolution of an enzyme that oxidized xylitol to L-xylulose would establish a new catabolic pathway for xylitol.

K. pneumoniae DM516 was previously constructed for the biological production of D-xylulose (Doten and Mortlock, submitted for publication). This strain constitutively synthesized the D-arabitol permease system and D-arabitol dehydrogenase but had a structural mutation in the D-xylulokinase of the D-arabitol pathway. A separate mutation prevented the synthesis of both the D-xylose isomerase and the D-xylulokinase of the D-xylose pathway.

We wished to eliminate ribitol dehydrogenase activity in strain DM516 by a mutation in the ribitol dehydrogenase structural gene. Since the inducer of the ribitol operon, D-ribulose, is an intermediate in the pathway, ribitol dehydrogenase-negative mutants and mutants that are unable to express the ribitol operon are indistinguishable. To overcome this identification problem, we first introduced a mutation to permit growth on D-arabinose. Strain DM601 was a D-arabinose-utilizing mutant of DM516. *K. pneumoniae* mutants able to grow on D-arabinose constitutively synthesize L-fucose isomerase (EC 5.3.1.3) and can employ this enzyme to isomerize D-arabinose to D-ribulose. This ability provides a mechanism for introducing the inducer of the ribitol operon without the activity of any ribitol catabolic

enzyme. The induction of D-ribulokinase then completes the D-arabinose catabolic pathway (20).

Strain DM601 was mutagenized, and ribitol-negative mutants were obtained. Two ribitol-negative mutants, strains DM704 and DM708, were still able to grow on D-arabinose, indicating that D-ribulokinase activity had been induced. This phenotype identified the inability of strains DM704 and DM708 to utilize ribitol as being due to a mutation in the ribitol dehydrogenase structural gene. Crude cell extracts of strains DM704 and DM708 grown in the presence of D-arabinose contained D-ribulokinase activity and no detectable level of ribitol dehydrogenase activity.

Strains DM704 and DM708 had the desired characteristics for use in directed-evolution experiments, as three separate mutations would be required to establish a xylitol pathway of the type previously observed. These strains also had the constitutive ability to transport xylitol and the ability to grow when L-xylulose was produced intracellularly.

Xylitol-positive mutants. Attempts to isolate xylitol-utilizing mutants directly on a solid medium were unsuccessful, although ca. 3.3×10^{10} cells of strain DM704 or DM708 were used in these experiments. However, after 12 days of incubation in a liquid medium containing xylitol as the potential carbon and energy source, growth occurred, and four independent xylitol-positive mutants were isolated. Strains DM801 and DM802 were xylitol-positive mutants of strain DM704, and strains DM803 and DM804 were xylitol-positive mutants of strain DM708. Crude cell extracts from each of four xylitol-grown mutants contained an NAD^+-dependent xylitol dehydrogenase activity, and an L-xylulokinase activity (Table 2), but no detectable D-xylulokinase or ribitol dehydrogenase activity. Therefore, the four xylitol-positive mutants appeared to catabolize xylitol through L-xylulose and not through D-xylulose. Polyacrylamide gel electrophoresis of xylitol-grown cell extracts under nondenaturing conditions showed that the four evolved dehydrogenases comigrated and were distinct from D-arabitol dehydrogenase (Fig. 3).

Transductional analysis of the evolved xylitol dehydrogenase structural gene. Phage P1CMc1r100 was used to isolate P1-sensitive mutants of strains DM516, DM803, and DM750. Strain DM750 was a D-arabitol dehydrogenase-negative mutant of strain DM708. Strains DM5169, DM8039, and DM7509 were P1-sensitive P1CMc1r100 lysogens of their respective parent strains. P1 transduction was used to investigate the genetic origin of the evolved xylitol dehydrogenase. Phage made on strain DM5169 or DM8039 was used to transduce strain DM7509 (Table 3). Analysis of the data showed that the genes coding for ribitol dehydrogenase and D-arabitol dehydrogenase are very closely linked. This has been shown in other *Klebsiella* species and in *E. coli* C (3, 23). It was possible to transfer the newly evolved xylitol dehydrogenase gene by transduction and to demonstrate that the evolved dehydrogenase was not an altered ribitol dehydrogenase or D-arabitol dehydrogenase and that the gene coding for the evolved dehydrogenase was not linked to the pentitol gene cluster. One xylitol-positive transductant, strain DM8889, was used in further investigations.

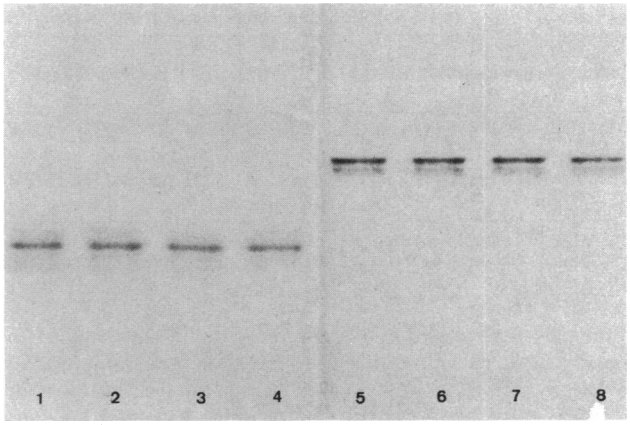

FIG. 3. Cell extracts from xylitol-positive mutants separated by nondenaturing polyacrylamide gel electrophoresis and stained for dehydrogenase activity. Lanes 1 to 4, Extracts from strains DM801, DM802, DM803, and DM804, respectively, stained for D-arabitol dehydrogenase activity. Lanes 5 to 8, Same extracts stained for xylitol dehydrogenase activity. Cells were grown on a xylitol-minimal salts medium.

TABLE 2. Enzyme activities of xylitol-utilizing mutants of *K. pneumoniae*

Strain	Growth substrate[a]	Sp act[b]	
		XDH	LXK
DM704	Xyt + CH	<0.005	<0.005
DM708	Xyt + CH	<0.005	<0.005
DM801	Xyt	0.063	0.182
DM802	Xyt	0.112	0.197
DM804	Xyt	0.079	0.167
DM803	Xyt	0.155	0.191
	CH	0.132	<0.005
DM8889	Xyt	0.178	0.194
	CH	0.155	<0.005

[a] Cells grown in a minimal salts-uracil medium plus the indicated carbon source. CH, Casein hydrolysate; Xyt, xylitol.

[b] Xylitol dehydrogenase activity (XDH) expressed as micromoles of NAD reduced per minute per milligram of protein; L-xylulokinase activity (LXK) expressed as micromoles of NADH oxidized per minute per milligram of protein.

Evolved xylitol pathway. Crude cell extracts of strains DM803 and DM8889 grown in the presence or absence of xylitol were assayed for xylitol dehydrogenase and L-xylulokinase activities (Table 2). Xylitol dehydrogenase activity was constitutively synthesized by both strains, whereas L-xylulokinase activity remained inducible. This result indicated that the genes coding for the dehydrogenase and the kinase were neither components of a single operon nor under the same regulatory control. The growth rates of several *K. pneumoniae* strains are shown in Table 4. Strain DM275, a xylitol-positive mutant of strain 261-5, used a constitutively synthesized ribitol dehydrogenase for the oxidation of xylitol. The growth rate on xylitol of strain DM8889, which possessed the evolved xylitol dehydrogenase, was higher than that of strain DM275.

Characterization of the evolved xylitol dehydrogenase. Xylitol dehydrogenase was purified 27-fold from strain DM8889 by DEAE-cellulose column chromatography and preparative polyacrylamide gel electrophoresis. One major and two minor bands were observed after nondenaturing electrophoresis of the purified enzyme preparation. Staining the gel for dehydrogenase activity showed that the major band was xylitol dehydrogenase. The substrate specificity of the en-

TABLE 3. Linkage of pentitol dehydrogenase genes as determined by P1 transduction

Donor strain[a]	Recipient strain	Selected marker	Unselected marker	Frequency of cotransduction[b]
DM5169 (ADH^c RDH$^+$)	DM7509 (ADH$^-$ RDH$^-$)	ADH^c	RDH$^+$	92 (332/360)
		RDH$^+$	ADH^c	92 (331/360)
DM8039 (XDH^c ADH^c RDH$^-$)	DM7509 (ADH$^-$ RDH$^-$)	ADH^c	XDH^c	0 (0/200)
		XDH^c	ADH^c	0 (0/120)

[a] ADH, D-Arabitol dehydrogenase; RDH, ribitol dehydrogenase; XDH, xylitol dehydrogenase; c, constitutive.
[b] Data are shown as percentages of cotransfer. The ratios in parentheses show data used to determine percentages.

zyme was determined by using nine polyols. Only xylitol and D-arabitol were substrates for NAD$^+$-dependent oxidation by the xylitol dehydrogenase. The rate of D-arabitol oxidation was only 1.5% that of xylitol oxidation, and no dehydrogenase activity was detected with mannitol, sorbitol, ribitol, L-arabitol, glycerol, 2,3-butanediol, or 1,2-propanediol as the substrate. The purified dehydrogenase catalyzed the NADH-dependent reduction of L-xylulose but not of D-xylulose, D-ribulose, D-xylose, or L-xylose. This difference indicated that the evolved enzyme was a xylitol-4-dehydrogenase and that no isomerase was involved in the xylitol pathway. The evolved dehydrogenase had a K_m for xylitol of 1.4 M and a molecular weight of 43,000 as determined by nondenaturing polyacrylamide gel electrophoresis.

DISCUSSION

The biochemical and genetic bases of the acquisition of a xylitol catabolic pathway have been studied as a model of catabolic evolution in several enteric bacteria, including *K. pneumoniae* PRL-R3. Interest in exploring the potential of *K. pneumoniae* PRL-R3 to evolve other xylitol catabolic pathways stems from investigations into xylitol pathways in *Erwinia* spp. Those *Erwinia* spp. examined oxidize xylitol at the C-4 position to produce L-xylulose by using an NAD$^+$-dependent xylitol-4-dehydrogenase (Doten and Mortlock, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K244, p. 217). Our knowledge of the metabolism of pentose and pentitol, the regulation and substrate specificities of pentose and pentitol catabolic enzymes, and the mechanisms involved in acquisitive catabolic evolution allows us to design experiments to evolve specific pathways and novel enzymatic activities. The strategy employed to determine whether *K. pneumoniae* PRL-R3 has the potential to evolve a second xylitol catabolic pathway was to create strains with a biochemical and genetic background that would direct selective pressure to evolve a xylitol-4-dehydrogenase. The strains constructed would require three separate mutations to recruit ribitol dehydrogenase and a D-xylulokinase and to evolve the xylitol catabolic pathway normally observed. The strains also constitutively synthesized D-arabitol dehydrogenase and the D-arabitol permease system. It was anticipated that D-xylulose would not evolve as an intermediate of the xylitol pathway. Therefore, no D-arabitol would be formed to induce the D-arabitol catabolic enzymes, necessitating the constitutive D-arabitol permease system. The constitutive D-arabitol dehydrogenase was supplied as a possible deregulated precursor of a xylitol-4-dehydrogenase in which structural mutations could lead to the xylitol dehydrogenase activity.

TABLE 4. Growth rates of *K. pneumoniae* mutants on pentitols

Strain	Growth substrate[a]	Mean generation time (h)
261-5	Ribitol	2.0
261-5	D-Arabitol	1.3
DM275	Xylitol	4.8
DM8889	Xylitol	3.1

[a] Cells were grown in a minimal salts-uracil medium plus the indicated pentitol.

A xylitol-4-dehydrogenase evolved in four separate experiments to allow growth on xylitol. The four evolved dehydrogenases comigrated on nondenaturing polyacrylamide gels, an indication that each dehydrogenase evolved from a common precursor. The mutation frequency for evolution of this xylitol dehydrogenase was less than 3×10^{-11}. It is probable that two mutations were required to establish the dehydrogenase activity, one of them regulatory, resulting in the constitutive synthesis of the novel dehydrogenase. It is difficult to speculate on the origin of the evolved dehydrogenase. Because of the differences in their regulation, it is unlikely that the evolved dehydrogenase and L-xylulokinase were components of a vestigial xylitol operon. Transductional analysis showed that the evolved dehydrogenase was not an altered form of either existing potential dehydrogenase and that the gene coding for the evolved dehydrogenase was not linked to the pentitol gene cluster. The substrate specificity of the dehydrogenase did not point to a possible origin, as the enzyme had best activity with xylitol as a substrate, slight activity with D-arabitol, and no activity with any other polyol tested. Cloning the gene coding for the evolved dehydrogenase may prove to be the best approach to investigating its origin. Using the cloned gene as a probe will allow the identification and isolation of its precursor, and this method can be used to screen for similar dehydrogenase precursors in other enteric bacteria.

A single regulatory mutation establishes a xylitol catabolic pathway in *K. pneumoniae* PRL-R3. This evolved pathway consists of existing ribitol and D-arabitol catabolic enzymes. Additional mutations increase the efficiency of xylitol utilization by altering the substrate specificity or regulation of the ribitol or D-arabitol enzymes (24). This study has shown that *K. pneumoniae* PRL-R3 has the evolutionary potential to evolve a second xylitol catabolic pathway that is as efficient as the previously evolved pathway. Evolution of this novel pathway appears to have required three mutations, and it is likely that additional mutations would improve the efficiency of the pathway. One of the general findings made in experimental attempts to evolve catabolic capabilities is that a bacterial strain responds to a specific stress in a specific manner. Repetition of selection experiments produces the same types of mutations in the same genes and in the same order (4, 8, 10, 15). One interpretation of this observation is that the evolutionary choices available are limited and steeply graded in terms of the adaptive possibilities that they can afford (9). Our ability to direct the evolution of a second xylitol catabolic pathway in *K. pneu-*

moniae PRL-R3 suggests that a genetic background may provide the potential to evolve several different degradative pathways for a novel compound and that the evolutionary option exercised is determined by existing pathways and regulatory mechanisms.

Altering pathways and regulation patterns allowed potential evolutionary options to be investigated under laboratory conditions. By constructing strains with other combinations of structural and regulatory mutations, it should be possible to explore other evolutionary options in *K. pneumoniae*. One possibility would be to direct the evolution of a *Lactobacillus*-type xylitol pathway in *Klebsiella* spp. Certain *Lactobacillus casei* strains employ a xylitol phosphotransferase system and a xylitol 5-phosphate dehydrogenase to metabolize xylitol (13). The fructose phosphotransferase system of *E. coli* K-12 also has activity on xylitol (21). If the *Klebsiella* fructose phosphotransferase system is similar, it should be possible to direct selective pressure to attempt to evolve a *Lactobacillus*-type xylitol pathway in *Klebsiella* spp.

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