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D-Arabitol Catabolic Pathway in *Klebsiella aerogenes*

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Klebsiella aerogenes strain W70 has an inducible pathway for the degradation of D-arabitol which is comparable to the one found in *Aerobacter aerogenes* strain PRL-R3. The pathway is also similar to the pathway of ribitol catabolism in that it is composed of a pentitol dehydrogenase, D-arabitol dehydrogenase (ADH), and a pentulokinase, D-xylulokinase (DXK). These two enzymes are coordinately controlled and induced in response to D-arabitol, the apparent inducer of synthesis of these enzymes. We obtained mutants which lacked a functional D-xylose pathway and were constitutive for the ribitol catabolic pathway. These mutants were able to grow on the unusual pentitol, xylitol, only if they contained the functional DXK of the D-arabitol pathway. This provided us with a specific selection technique for DXK^+ transductants. As in *A. aerogenes*, mutants constitutive for ADH were able to use this enzyme to convert the hexitol D-mannitol to D-fructose. With mutants blocked in the normal D-mannitol catabolic pathway, growth on D-mannitol became a test for ADH constitutivity. Growth of such mutants on xylitol, D-arabitol, and D-mannitol was utilized to classify transductants in mapping, by transductional analysis, the loci involved in D-arabitol utilization. Three-point crosses gave the order *dalK-dalD-dalC*, where *dalK* is the DXK structural gene, *dalD* is the ADH structural gene, and *dalC* is a regulatory site controlling synthesis of both enzymes.

Of the four isomeric pentitols, ribitol, D-arabitol, L-arabitol, and xylitol, only the first two are abundant in nature. Most of the previous work on catabolism of these compounds was done with various *Aerobacter aerogenes* strains (3, 10), organisms which would probably be classified now as *Klebsiella* or *Enterobacter* species. The organism of choice in this laboratory had been *A. aerogenes* strain PRL-R3, but the lack of a suitable genetic exchange system encouraged us to shift our work to a study of the closely related *Klebsiella aerogenes* strain W70.

The pathway in *K. aerogenes* W70 for catabolism of ribitol (2) is composed of at least two enzymes, ribitol dehydrogenase (EC 1.1.1.56; RDH), which oxidizes ribitol to D-ribulose, and D-ribulokinase (EC 2.7.1.47; DRK), which phosphorylates D-ribulose, a situation directly analogous to that found in *A. aerogenes*. The genetic determinants of that pathway, in addition to closely linked sites for the two structural proteins RDH and DRK, include at least one closely linked regulatory site controlling synthesis of both enzymes.

This paper compares the pathway for D-arabitol degradation in *K. aerogenes* strain W70 with that reported for *A. aerogenes* strain PRL-R3 and, in addition, deals with studies on the genetic determinants of this pathway in *K. aerogenes*.

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MATERIALS AND METHODS

Bacterial strains and cultural conditions. *K. aerogenes* strain W70 and the transducing phage PW52 were obtained from J. F. Wilkinson, Department of General Microbiology, University of Edinburgh, Scotland (5). Media and cultural conditions were those described previously (2). Preparation of cell free extracts has also been described previously (9).

Enzymatic assays. Dehydrogenase activities were determined in a continuous spectrophotometric assay monitoring reduced nicotinamide adenine dinucleotide utilization in the presence of D-ribulose or D-xylulose (10). D-Xylulokinase (DXK) was measured in a continuous spectrophotometric assay by determining adenosine diphosphate formation with the pyru-

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vate kinase-lactic acid dehydrogenase system (1). D-Xylose isomerase (DXI; EC 5.3.1.5) activity was measured by determining the rate of keto sugar production from the corresponding aldo sugar (1).

Chemicals. D-Xylulose was prepared chemically by refluxing D-xylulose with dry pyridine (6). D-ribulose was prepared from ribitol with whole cells of a mutant of *A. aerogenes* PRL-R3 (7).

The following chemicals were obtained commercially: casein hydrolysate (vitamin-free, salt-free) from Nutritional Biochemicals Corp., Cleveland, Ohio; adenosine 5'-triphosphate, ribitol, xylitol, D-arabitol, D-xylose and lactic acid dehydrogenase, and pyruvate-kinase combination (for kinase assays) from Sigma Chemical Co., St. Louis, Mo.; and nutrient broth and agar from Difco Laboratories, Detroit, Mich.

RESULTS

Induction of the enzymes for D-arabitol catabolism. *A. aerogenes* strain PRL-R3 catabolizes D-arabitol by oxidation to D-xylulose followed by phosphorylization to D-xylulose-5-phosphate (Fig. 1). The corresponding aldopentose, D-xylose, is isomerized to D-xylulose and then phosphorylated at the C_5 position. Table 1 shows the activity of D-arabitol dehydrogenase (ADH), DXK, and DXI in cell-free extracts prepared from cells of *K. aerogenes* W70 which had been grown on casein hydrolysate or on casein hydrolysate supplemented with D-arabitol or D-xylose. Significant levels of both ADH and DXK were induced only in the presence of D-arabitol, whereas incubation with D-xylose resulted in the induction of DXI and DXK activities. Significant levels of these enzymes were not found when ribitol, xylitol, L-arabitol, or D-mannitol were added as supplement.

Wilson and Mortlock (9) have reported two separate DXKs in *A. aerogenes*, one induced by D-arabitol and the other induced by D-xylose. Differences in the stability of the DXK activities induced by either D-xylose or D-arabitol indicate the D-xylose and D-arabitol pathways in *K. aerogenes* also use separate kinases (as shown in Fig. 1), rather than a single structural protein under dual regulatory control. As found by Wilson and Mortlock in *A. aerogenes* PRL-R3, the D-arabitol-induced kinase in *K. aerogenes* W70 was cold labile, losing essentially all of its activity within 48 h when stored at 0 C, but still maintaining about 50% of its original activity when stored for the same time period at 22 C. D-Xylose-induced enzyme, however, stored under the same conditions retained about 50% of its original activity both at 0 and 22 C. This suggested the possibility of two distinct structural DXKs in *K. aerogenes* as demonstrated in *A. aerogenes*.

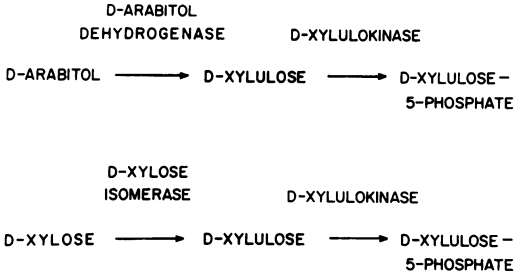

FIG. 1. *Pathways for the degradation of D-xylose and D-arabitol.*

TABLE 1. *Induction of the enzymes of the D-xylose and D-arabitol catabolic pathways*

Carbohydrate in medium	Enzyme activities[a]		
	D-Xylose isomerase	D-Arabitol dehydrogenase	D-Xylulokinase
None	<0.02[b]	<0.01	<0.01
D-Arabitol	<0.02	1–5[c]	1–5
D-Xylose	0.05–1.3	<0.01	0.5–2.5

[a] Enzymatic activities, expressed as micromoles per milligram of protein per minute, were obtained from cell-free extracts of cells grown on 1% casein hydrolysate supplemented with 0.5% of the indicated carbohydrate.

[b] < indicates activity not detectable but less than the indicated value.

[c] The range of values indicates the high and low values from three independent experiments.

If this were true, it would have been difficult to isolate a DXK-negative mutant directly from the wild-type organism. To test this concept, we isolated mutants for their inability to grow with D-xylose as the sole carbon and energy source. After the cells were grown on casein hydrolysate in the presence of D-xylose, cell-free extracts were prepared and assayed for DXI and DXK activities. Two phenotypes were identified. One type of mutant produced neither isomerase nor kinase activity upon incubation with D-xylose. The second type of mutant showed no detectable isomerase activity but produced normal levels of kinase activity, indicating that D-xylose functioned as the apparent inducer of the DXK of the D-xylose catabolic pathway. The activities for some of these mutant strains are shown in Table 2. One of the first types of double-negative mutant, strain 7a, was used as a parent strain for the isolation of D-arabitol-negative mutants. When incubated on casein hydrolysate supplemented with D-arabitol and assayed for dehydrogenase and kinase activities, these new mutants fell into three classes (Table 3). One type of mutant, *dalD2*, produced kinase but not dehydrogenase activity; a second

class, *dalK3*, produced dehydrogenase but not kinase activity; and the third class of mutant produced neither dehydrogenase nor kinase activity. These data are similar to those reported for *A. aerogenes* strain PRL-R3 (9). Mutant strains unable to elicit dehydrogenase activity but able to elicit kinase activity are believed to be structural mutations in the gene coding for ADH, whereas mutants unable to produce kinase but able to elicit dehydrogenase activity are believed to be DXK structural gene mutations. Our ability to elicit kinase activity in mutants lacking dehydrogenase activity indicated that D-arabitol, rather than an intermediate of the pathway, was the apparent inducer of the DXK of the D-arabitol pathway.

TABLE 2. *D-Xylose isomerase and D-xylulokinase activities found in the D-xylose-negative mutant strains*

Strain	Inducer	Activities[a]	
		D-Xylose isomerase	D-Xylulokinase
K. aerogenes W70	None	<0.02[b]	0.08
	D-Xylose	0.1	0.8
D-Xylose-negative mutants			
7A	D-Xylose	<0.02	0.03
8B	D-Xylose	<0.02	3.2
8B	None	<0.02	0.1

[a] Enzymatic activities, expressed as micromoles per milligram of protein per minute, were obtained from cell-free extracts of cells grown on 1% casein hydrolysate supplemented with 0.5% D-xylose where indicated.

[b] < indicates activity not detectable but less than the indicated value.

Selection and scoring of constitutive mutants. Two methods have been described in the literature for the isolation of *A. aerogenes* mutants constitutive for ADH activity. The first method is based on the reduction of 2,3,5-triphenyl tetrazolium chloride by uninduced cells incubated with D-arabitol and chloramphenicol (4). The second method is based on the concept that ADH can catalyse the oxidation of D-mannitol to D-fructose. As reported by Tanaka et al. (8), mutants of *A. aerogenes* which have lost the ability to degrade D-mannitol can grow by utilizing D-mannitol as the sole carbon and energy source if they become constitutive for ADH (Fig. 2). Since this mechanism, utilizing the dehydrogenase-constitutive alternative for growth on D-mannitol, would allow us to select and identify D-arabitol dehydrogenase constitutive mutants, we examined this system in *K. aerogenes* W70. Seven out of eight independent revertants of D-mannitol-negative mutants were constitutive for ADH, indicating a phenotypic reversion to the D-mannitol-positive phenotype in a manner similar to that found in *A. aerogenes*.

We were able to construct, in strains lacking a functional DXK, mutants which were constitutive for ADH and still phenotypically D-mannitol positive. These data were in agreement with those of Tanaka et al. (8), that the elevated ADH levels in ADH-constitutive mutants allow growth on D-mannitol by the enzymatic conversion of D-mannitol to D-fructose or of D-mannitol-1-phosphate to D-fructose-6-phosphate, and that D-arabitol dehydrogenase was active with either D-mannitol or D-mannitol-1-phosphate as substrate. Therefore, we could select and score dehydrogenase-constitutive mutants,

TABLE 3. *D-Arabitol dehydrogenase and D-xylulokinase activities found in mutant strains*[a]

Strain	Inducer	Activity	
		D-Arabitol dehydrogenase	D-Xylulokinase
K. aerogenes W70	None	<0.05[b]	<0.01
K. aerogenes W70	D-Arabitol	0.8-4.0[c]	0.5-2.0
Constitutive (*dalC16*)	None	1.0-4.0	0.5-4.0
D-Arabitol dehydrogenase (*dalD2*) negative	D-Arabitol	<0.05	0.4-1.2
D-Xylulokinase negative (*dalK3*)	D-Arabitol	1.0-3.0	<0.01
Simultaneous loss of both enzyme activities (*dal-22*)	D-Arabitol	<0.05	<0.01

[a] Enzymatic activities, expressed as micromoles per milligram of protein per minute, were obtained from cell-free extracts of cells grown on 1% casein hydrolysate supplemented with 0.5% D-arabitol where indicated.

[b] < indicates activity not detectable but less than the indicated value.

[c] Range of values from at least three independent experiments.

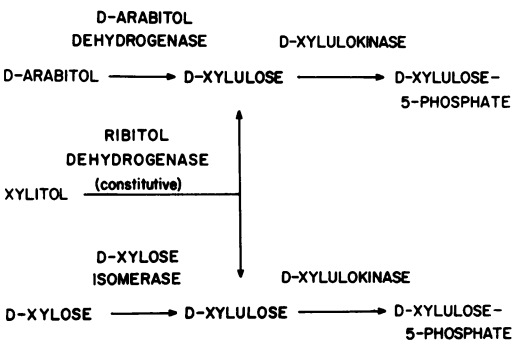

FIG. 2. *Pathways for the degradation of xylitol.*

in strains appropriately marked with a lesion in the D-mannitol catabolic pathway, by their ability to grow on D-mannitol. When a stable D-mannitol-negative mutant was isolated from a ADH-negative strain and transduced to become D-arabitol positive with phage from a ADH-constitutive donor, all of the dehydrogenase-constitutive transductants were phenotypically D-mannitol positive. Six independently isolated ADH-constitutive mutants isolated by the tetrazolium spray test (4) and 37 independently isolated dehydrogenase-constitutive mutants isolated from D-mannitol plates were tested for constitutive synthesis of both the dehydrogenase and the kinase. All 43 mutants tested were constitutive for both dehydrogenase and kinase activities even though the screening procedures were selective for only the dehydrogenase-constitutive phenotype, indicating that the dehydrogenase and kinase induced in response to D-arabitol are coordinately controlled.

Growth on xylitol. *K. aerogenes* is able to grow on xylitol if it has an *rbtC* mutation to constitutive synthesis of RDH. This permits conversion of xylitol to D-xylulose (2). To determine the origin(s) of the DXK activity required for growth on xylitol, we chose a derivative of the D-xylose-negative mutant 7A, which contained the D-mannitol-1, *dalD* (ADH negative), and *rbtC13* mutations. This strain was used as a parent to select xylitol-negative mutants. The resultant xylitol-negative mutants were of two distinct types. The first was no longer RDH constitutive and, therefore, could not oxidize xylitol to D-xylulose, whereas the second was still RDH constitutive but no longer produced DXK when grown on casein hydrolysate supplemented with D-arabitol. The latter mutant was capable of oxidizing xylitol to D-xylulose (using the constitutive ribitol dehydrogenase) but could not grow with xylitol as the substrate since it had lost the ability to synthesize both of its DXKs.

When an *rbtC13* donor which was capable of growth on D-xylose and D-arabitol was used as donor to transduce the latter mutant to xylitol positive, three classes of transductants were found. The first class (134 transductants) was D-xylose positive, D-arabitol negative and, as expected, produced kinase and isomerase activities in response to D-xylose but no dehydrogenase or kinase in response to D-arabitol. The second class (280 transductants) was D-arabitol positive, D-xylose negative, and produced dehydrogenase and kinase in response to D-arabitol but no kinase or isomerase in response to D-xylose. The first class had obtained, by transduction, the DXK of the D-xylose pathway, whereas the second class had obtained the DXK of the D-arabitol pathway. The third class (16 transductants) was D-xylose negative, D-arabitol negative, but produced DXK but no dehydrogenase in response to D-arabitol and no kinase or isomerase in response to D-xylose. This class presumably had obtained the kinase, but not the dehydrogenase, of the D-arabitol pathway from the donor. In the same cross, we selected independently for growth on D-xylose and D-arabitol. All of both of these latter classes of transductants were xylitol positive, and representative transductants produced the expected enzymatic activities.

These data demonstrate that both of the DXKs are sufficient to permit growth on xylitol in RDH-constitutive mutants (Fig. 2). This is in agreement with recently published data (9) for *A. aerogenes*.

Genetic determinants of growth on D-arabitol. Basic two-point crosses demonstrated that all of the determinants of the D-arabitol catabolic pathway thus far examined were closely linked genetically. With *dalC* mutants (constitutive for ADH and DXK) as donors in each case and D-mannitol-negative, D-arabitol-negative, mutants as recipients, we selected for D-arabitol-positive phenotypes and transferred these transductants to D-mannitol plates to test for ADH constitutivity. In each case, a co-transduction frequency for the D-arabitol-positive and the *dalC* mutations of over 90% was found.

Figure 3 represents the type of three-point cross used to order the mutations in the D-arabitol pathway. The recipients were D-xylose negative, D-mannitol negative, RDH constitutive, ADH negative, and DXK negative. The donor strain contained the same D-xylose, D-mannitol, and *rbtC13* lesions as the recipient but was constitutive for both ADH and DXK activities.

In the type of cross represented by Fig. 3, DXK-positive transductants (*dalK*$^+$) were se-

lected on xylitol and picked to D-arabitol and D-mannitol plates. These *dalK*$^+$ transductants resulted from crossovers which originated to the left of *dalK4* (Fig. 3) and terminated to the right of *dalK4* in region A, B, or C. Kinase-positive, dehydrogenase-negative inducible transductants (*dalK*$^+$, *dalD2*, *dalC*$^+$), which received only the *dalK*$^+$ genotype from the donor (crossovers terminating in region A), were unable to grow on D-arabitol or D-mannitol. Cells which were *dalK*$^+$, *dalD*$^+$, and *dalC*$^+$ received the *dalK*$^+$ and the *dalD*$^+$ genotypes from the donor (crossovers terminating in region B) and could grow on xylitol and D-arabitol, but not on D-mannitol. Grown on D-mannitol required, in addition to *dalK*$^+$ and *dalD*$^+$ genotypes, a *dalC16* mutation (from crossovers terminating in region C). Transductants which grew on xylitol but not D-arabitol were grown in casein hydrolysate medium with and without D-arabitol. In all of the transductants, kinase but not dehydrogenase activity was present in extracts prepared from cells grown in the presence of D-arabitol, but neither activity could be detected when the cells had been grown on casein hydrolysate alone. Thus, the cells had been transduced only for *dalK*$^+$, indicating the gene order shown in Fig. 3. If the *dalK* site were between the *dalD* and the *dalC* sites or to the right of the *dalC* site as drawn, we would have expected to find the *dalK*$^+$ *dalC16* *dalD2* genotype, which was not observed.

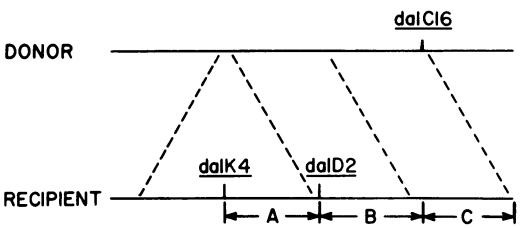

FIG. 3. *Diagram of the crosses which gave the data in Table 4, where crossovers originating to the left of the selected marker dalK4 terminated to the right of dalK4 in region A, B, or C.*

The possibility that we simply did not screen a sufficient number of *dalK*$^+$ transductants is precluded by the fact that we did find, in addition to *dalK*$^+$ *dalD*$^+$ *dalC16*, reasonable numbers of both *dalK*$^+$ *dalD*$^+$ *dalC*$^+$ and *dalK*$^+$ *dalD2* *dalC*$^+$ genotypes from crossovers terminating in regions A and B, indicating that both the *dalD2* and the *dalC16* mutations used were separable by transduction from the *dalK4* mutations (Table 4). Table 4 demonstrates that essentially the same results were obtained when *dalC17*, *dalC18*, or *dalC19* mutations were used as donor in similar crosses. Figure 4 is our representation of the region of the *K. aerogenes* W70 genome coding for the enzymes of the D-arabitol catabolic pathway. The brackets correspond to the limits of these sites as determined by the two- and three-point crosses. Three-point crosses with *dal-21* and *dal-22* are not feasible with the mutants available at this time.

DISCUSSION

A. aerogenes strain PRL-R3 has separate inducible pathways for the degradation of the two naturally occurring pentitols, ribitol and D-arabitol. We have already presented evidence for a ribitol catabolic pathway in *K. aerogenes* strain W70, composed of two enzymes, RDH and DRK, and a regulatory site, *rbtC*(2).

We have again taken advantage of the information gleaned from *A. aerogenes* and applied it to a study of the second pentitol catabolic pathway in *K. aerogenes* strain W70.

D-Arabitol, in *K. aerogenes* W70 as in *A. aerogenes* PRL-R3, is degraded by a pathway that is similar to the catabolic pathway for ribitol, the other commonly occuring pentitol. Both pentitols are initially oxidized to the

TABLE 4. *Results of three-point crosses*[a]

dalC mutant	Total *dalK*$^+$ scored	No. of transductants			
		dalK$^+$ *dalD2* *dalC*$^+$	*dalK*$^+$ *dalD2* *dalC*$^-$	*dalK*$^+$ *dalD*$^+$ *dalC*$^+$	*dalK*$^+$ *dalD*$^+$ *dalC*$^-$
17	474	10	0	4	460
19	329	3	0	9	317
16	356	3	0	7	346
18	407	2	0	10	395

[a] Donor: mannitol-1 *dalC* mutants indicated. Recipient: mannitol-1 *dalD2* *dalK4*. Transductants (*dalK*$^+$) were selected on xylitol and tested for growth on D-arabitol (*dalK*$^+$ *dalD*$^+$) and mannitol (*dalK*$^+$ *dalD*$^+$ *dalC*$^-$). *dalK*$^+$ *dalD*$^-$ recombinants were grown and assayed for constitutive synthesis of D-xylulokinase to identify inducible (*dalK*$^+$ *dalD2* *dalC*$^+$) and constitutive (*dalK*$^+$ *dalD2* *dalC*$^-$) transductants lacking a functional D-arabitol dehydrogenase.

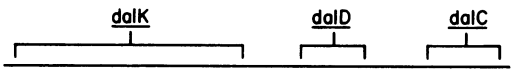

FIG. 4. *dal region in Klebsiella aerogenes strain W70.*

corresponding 2-keto sugars, D-ribulose in the case of ribitol and D-xylulose in the case of D-arabitol. The pentuloses are then phosphorylated by the distinct pentulokinases, DRK and DXK, respectively.

When we examined the genetic determinants of growth on ribitol, we demonstrated two closely linked sites for the structural genes for RDH and DRK and a closely linked control site. Mutations in the control site, *rbtC*, were identified by growth on xylitol. In the case of D-arabitol catabolism, we have also shown two closely linked sites for the structural genes for ADH and DXK, *dalD* and *dalK*, respectively, and a closely linked control site, *dalC*. In the crosses, we used donors and recipients which contained the same lesions to the D-mannitol-negative phenotype. This allowed us to accurately identify ADH-constitutive mutations resulting from lesions in the closely linked *dalC* site by their ability to grow at the expense of D-mannitol.

In our study of ribitol catabolism, we found that D-ribulose, an intermediate of ribitol catabolism, was the apparent inducer of RDH and DRK activities. In the case of the D-arabitol pathway, the corresponding dehydrogenase and kinase were induced in response to D-arabitol rather than an intermediate of the D-arabitol catabolic pathway. We can rationalize this difference by remembering the similarity of the corresponding alcohols and keto sugars of these two pathways. To insure independent control, such a difference in inducers would be beneficial. In addition, D-arabitol is oxidized to D-xylulose, which is an intermediate of not only the D-arabitol pathway but also of the D-xylose pathway.

In *A. aerogenes*, D-arabitol has also been shown to be the inducer of the dehydrogenase and kinase activities and, in that system, D-xylose has been shown to be the inducer of its catabolic pathway (9). Similarly, in *K. aerogenes*, the D-xylose pathway also appears to use the initial compound D-xylose as the inducer of that pathway, as indicated by the ability of DXI-negative mutants to elicit DXK activity in response to D-xylose in the growth media. Another similarity between these two strains is that the DXK induced by D-arabitol in both organisms is cold sensitive, losing its activity more rapidly at 0 C than at 15 or 22 C, whereas the DXK induced by both organisms in response to D-xylose is not cold sensitive.

Thus, both *A. aerogenes* and *K. aerogenes* degrade D-arabitol, ribitol, and D-xylose by inducible pathways which are similar in terms of the enzymes involved and the apparent inducers of these enzymes. Subsequent work will concentrate on *K. aerogenes*, where the available genetic exchange system allows more extensive studies.

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