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Ribitol Catabolic Pathway in *Klebsiella aerogenes*

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In *Klebsiella aerogenes* W70, there is an inducible pathway for the catabolism of ribitol consisting of at least two enzymes, ribitol dehydrogenase (RDH) and D-ribulokinase (DRK). These two enzymes are coordinately controlled and induced in response to D-ribulose, an intermediate of the pathway. Whereas wild-type *K. aerogenes* W70 are unable to utilize xylitol as a carbon and energy source, mutants constitutive for the ribitol pathway are able to utilize RDH to oxidize the unusual pentitol, xylitol, to D-xylulose. These mutants are able to grow on xylitol, presumably by utilization of the D-xylulose produced. Mutants constitutive for L-fucose isomerase can utilize the isomerase to convert D-arabinose to D-ribulose. In the presence of D-ribulose, RDH and DRK are induced, and such mutants are thus able to phosphorylate the D-ribulose by using the DRK of the ribitol pathway. Derivatives of an L-fucose isomerase-constitutive mutant were plated on D-arabinose, ribitol, and xylitol to select and identify mutations in the ribitol pathway. Using the transducing phage PW52, we were able to demonstrate genetic linkage of the loci involved. Three-point crosses, using constitutive mutants as donors and RDH^-, DRK^- double mutants as recipients and selecting for DRK^+ transductants on D-arabinose, resulted in DRK^+ RDH^+-constitutive, DRK^+ RDH^+-inducible, and DRK^+ RDH^--inducible transductants but no detectable DRK^+ RDH^- constitutive transductants, data consistent with the order *rbtC-rbtD-rbtK*, where *rbtC* is a control site and *rbtD* and *rbtK* correspond to the sites for the enzymes RDH and DRK, respectively.

Early work on the catabolism of pentoses and pentitols by *Aerobacter aerogenes* strain PRL-R3 showed that the naturally occurring pentoses are utilized by a mechanism involving isomerization to the corresponding 2-keto sugar and then phosphorylation to the ketopentose-5-phosphate (15). The exception is D-ribose, which is phosphorylated and then isomerized. The two naturally occurring pentitols, ribitol and D-arabitol, are oxidized to the corresponding 2-ketopentuloses by ribitol dehydrogenase (RDH; EC 1.1.1.56) or D-arabitol dehydrogenase (EC 1.1.1.11), respectively, and then phosphorylated to the corresponding pentulose-5-phosphate (Fig. 1; 22). The two enzymes of the ribitol catabolic pathway, RDH and D-ribulokinase (DRK; EC 2.7.1.47), have been shown to be under coordinate control, with the intermediate in the pathway, D-ribulose, as the apparent inducer (3). The two enzymes of the D-arabitol pathway, D-arabitol dehydrogenase and D-xylulokinase (EC 2.7.1.17), have also been reported to be under coordinate control, with the initial substrate, D-arabitol, as the apparent inducer (21). Unfortunately, no suitable genetic exchange system exists for *A. aerogenes* PRL-R3 which would permit a study of the genetic basis of the catabolism of these 5-carbon sugars.

In 1969, McPhee et al. reported a transduction system for *Klebsiella aerogenes* strain W70 (13). We were able to confirm their report of transduction in *K. aerogenes* W70 by bacteriophage PW52, and preliminary studies showed that *K. aerogenes* W70 was similar to *A. aerogenes* PRL-R3 in respect to its catabolism of the naturally occurring C_5 sugars. In addition, the mutations permitting growth on some of the unnatural C_5 sugars were found to be of a similar nature to those reported for the PRL-R3 strain of *A. aerogenes*. We therefore have directed our attention to the catabolism of the C_5 sugars by this strain of *K. aerogenes* in order to study the regulation and genetic determinants of the enzymes of these catabolic pathways.

In this paper, we examine the inducible pathway for the catabolism of ribitol by *K. aerogenes* W70 and show its similarity in terms of the enzymes involved, and their regulation,

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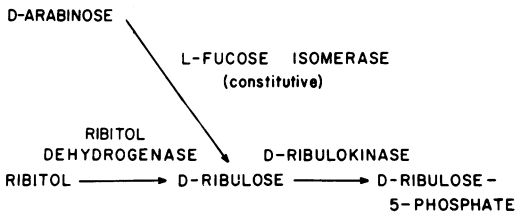

FIG. 1. *Pathways for the degradation of ribitol and D-arabinose.*

to the pathway studied in *A. aerogenes* PRL-R3 (3, 22). Some of the genetic determinants of the components of this pathway will also be considered.

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MATERIALS AND METHODS

Chemicals. D-Ribulose was synthesized enzymatically by the oxidation of ribitol (18), isolated as the *o*-nitrophenyl-hydrazone derivative (5), and regenerated for use by the procedure of Muller et al. (17). L-Xylulose and L-ribulose were prepared chemically from the corresponding aldopentose by refluxing with dry pyridine (16). L-Fuculose was prepared by the enzymatic isomerization of L-fucose (8).

D-Arabinose, L-fucose, ribitol, xylitol, adenosine 5'-triphosphate, and lactic acid dehydrogenase-pyruvate kinase mixture (for kinase assays) were purchased from Sigma Chemical Co., St. Louis, Mo.; nutrient broth and agar were purchased from Difco Laboratories, Detroit, Mich.

Bacterial strains and cultural conditions. *K. aerogenes* W70 and the transducing phage PW52 were obtained from J. F. Wilkinson, Department of General Microbiology, University of Edinburgh, Scotland. Mutants were derived in this laboratory by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.) by the procedure of Adelberg et al. (1), modified to 300 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml final concentration and 1 h of exposure. Penicillin enrichments were basically by the method of Davis (6), with the penicillin concentration increased to 5 mg/ml and added only after growth had begun. Stock cultures were maintained in 5-ml volumes of NBU broth (see below) in screw-cap Pyrex tubes (16 by 100 mm). Uninfected stock cultures were transferred every 2 months. Cultures infected with the transducing phage PW52 were transferred every 4 weeks.

For enzymatic assays, cells were grown on minimal media (18) supplemented with 0.5% carbohydrate and/or 0.5% casein hydrolysate (vitamin-free, salt-free, acid-hydrolyzed, Nutritional Biochemicals, Cleveland, Ohio) at 37 C. NBU medium contained 8 g of nutrient broth (Difco Laboratories, Detroit, Mich.) and 50 mg of uracil in 1 liter of distilled water. Agar (Difco) was used at 1.5% for base agar plates and 0.75% for soft agar overlays. All broth cultures were grown at 37 C on a New Brunswick model VS rotary shaker at 250 rpm.

Preparation of cell-free extracts. Cells were pelleted by centrifugation at 4,340 $\times$ *g* and washed once in 50% the original volume of distilled water. The washed cells were pelleted by centrifugation at 4,340 $\times$ *g*, suspended in 0.4 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.5), and transferred to polyethylene tubes. The tubes were placed in the cup of a Raytheon 10 kc magnetoconstrictive oscillator (Raytheon Co., South Norwalk, Conn.) containing about 30 ml of water, and the cells were disrupted by sonic treatment for 15 min. Remaining cells and cellular debris were removed by centrifugation at 35,000 $\times$ *g* for 15 min (21).

Enzymatic assays. DRK and L-ribulokinase (EC 2.7.1.16) were determined, in a continuous spectrophotometric assay, by measuring adenosine diphosphate formation with the pyruvate kinase-lactic acid dehydrogenase system (2). When dehydrogenase activity, which interfered with the spectrophotometric assay, was high, the rate of disappearance of the pentulose was determined (11). L-Fucose isomerase (D-arabinose isomerase, EC 5.3.1.3) and L-arabinose isomerase (EC 5.3.1.4) activities were measured by determining the rate of keto sugar production from the corresponding aldo sugar (2). L-Fucose isomerase activity was also determined spectrophotometrically (19). RDH activity was determined in a continuous spectrophotometric assay by monitoring reduced nicotinamide adenine dinucleotide utilization in the presence of D-ribulose (22).

Selection of constitutive mutants. Constitutive mutants were selected by the method of Lin et al. (12), by testing for the ability of uninduced cells to reduce 2,3,5-triphenyl tetrazolium chloride when given ribitol as a substrate in the presence of chloramphenicol, or by selecting mutants capable of utilizing xylitol as a sole carbon and energy source (14). Both techniques were selective only for constitutive RDH production and were effective in mutants lacking demonstrable DRK activity.

Phage propagation and assay. Cultures of *K. aerogenes* W70 were infected with bacteriophage PW52 by mixing 5 $\times$ 10^8 exponentially growing cells and 10^9 phage in 4.5 ml of NBU broth in a Pyrex screw-cap tube (16 by 100 mm). The mixtures were incubated on a rotary shaker at 250 rpm for 6 to 12 h, and the cultures were streaked on NBU agar plates. After two single-colony isolations, broth cultures were prepared and tested for production of PW52 in the phage assay described below.

Phage lysates were prepared from infected clones by initiating cultures in NBU broth in Pyrex screw-cap tubes (16 by 100 mm). When the cultures reached an optical density of 0.6 to 0.8, as determined on a Spectronic 20 colorimeter, the cells were exposed to ultraviolet radiation 43 cm from two 15-W General Electric germicidal bulbs in a 100-mm glass petri dish on a rotary shaker at 125 rpm. After irradiation, the

cells were diluted 1:10 into prewarmed NBU broth and incubated with shaking at 37 C. When lysis was evident (2.5 to 3 h), the cells were removed by centrifugation at 7,740 × *g* for 20 min, and any remaining cells were removed from the lysate by passage of the lysate through a 0.45-μm membrane filter (Millipore Corp.). The resulting lysates routinely titered 10^{10} to 5×10^{10} infectious particles per ml.

The phage were assayed by adding 0.1 ml of cells in the exponential growth phase and 0.1 ml of the appropriate dilution of phage to 2.0 ml of molten NBU agar (0.75% agar). The mixture was agitated and layered into a petri dish containing 25 to 36 ml of fresh NBU agar. The plates were incubated at 37 C for at least 6 h, at which time plaques were evident. Incubation could be continued for at least 24 h before the plaques were obscured by overgrowth of the lawn.

Transductions. Recipient cultures were infected and cloned, as described above, prior to the transduction. A culture was initiated from a fresh refrigerated NBU broth culture of the infected mutant as a 1:10 dilution into 4.5 ml of fresh NBU broth in a Pyrex screw-cap tube (16 by 100 mm). The cultures, after incubation for 3 h at 37 C as described above, were in exponential growth and had an optical density of 0.6 to 0.9 as determined in a Bausch and Lomb Spectronic 20 colorimeter. The cell titer at this time was in the range of 0.8×10^9 to 1.3×10^9 colony-forming units per ml. The cells were pelleted by centrifugation at 4,340 × *g* at ambient temperature, the supernatant fluid was removed, and the cells were resuspended in phage lysate. After adsorption at 37 C for 20 min, the mixture was added to 1.5 ml of liquid salts agar to 0.75% agar and overlaid onto appropriate selective plates. In the range of cell concentrations described above, there was only a slight increase (30%) in transduction frequency from the lower cell titer to the higher cell titer. For this reason, we routinely determined that the optical density was in the acceptable range but did not determine viable cell counts for determinations of multiplicity of infection.

RESULTS

Induction of the enzymes for ribitol catabolism. Table 1 shows the activity of RDH and DRK present in cell-free extracts obtained from cells of *K. aerogenes* strain W70 which had been grown on casein hydrolysate or casein hydrolysate supplemented with one of the four pentitols. Significant levels of these enzymes were induced only in the presence of ribitol. A number of mutants were isolated which had lost the ability to utilize ribitol as a sole carbon and energy source. When these mutant strains were grown on casein hydrolysate in the presence of ribitol and cell-free extracts were prepared and assayed for the presence of the dehydrogenase and kinase activities, two types of response were observed (Table 1). One type of mutant possessed no detectable levels of either dehydrogenase or kinase when grown under these conditions, whereas the other type possessed dehydrogenase but not kinase activity.

TABLE 1. *Induction of ribitol dehydrogenase and* D-*ribulokinase activities*

Organism	Pentitol	Enzymatic activities[a] Ribitol dehydrogenase	Enzymatic activities[a] D-Ribulokinase
Klebsiella aerogenes strain W70	None	<0.01[b]	<0.01
	Ribitol	1-5[c]	0.5-2
	D-Arabitol	<0.01	<0.01
	Xylitol	<0.01	<0.01
	L-Arabitol	<0.01	<0.01
Ribitol-negative mutants			
A	Ribitol	<0.01	<0.01
B	Ribitol	2	<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, where indicated, with 0.5% of the indicated pentitol.

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

[c] Ranges of activity represent high and low values obtained in at least three separate experiments.

These data are similar to those previously reported for *A. aerogenes* strain PRL-R3 (3). It was shown that D-ribulose, rather than ribitol, functioned as inducer of the enzymes for ribitol catabolism and that cells lacking dehydrogenase activity were unable to oxidize ribitol to D-ribulose and, thus, unable to induce D-ribulokinase activity (3). It has also been reported that mutants of *A. aerogenes* PRL-R3, constitutive for L-fucose isomerase, are capable of isomerizing D-arabinose to D-ribulose (4, 20). This results in the induction of both enzymes of the ribitol catabolic pathway, RDH and DRK (3).

To determine whether a similar situation existed for *K. aerogenes* W70, mutants capable of growth with D-arabinose as the carbon and energy source were selected and assayed for constitutive L-fucose isomerase activity. The results of assays using one such mutant are shown in Table 2. This mutant was constitutive for the isomerase, synthesizing, when grown on casein hydrolysate alone, isomerase activity similar to that induced in the parent strain by L-fucose. We were able to demonstrate, in crude extracts of the L-fucose isomerase-constitutive mutants, the production of a ketopentose from D-arabinose. The ketopentose was identified as D-ribulose by the rate of color development in the cysteine carbizole test for ketopentoses (7) and its ability to serve as a substrate for RDH.

TABLE 2. *Enzymatic activities associated with growth on* D-*arabinose*

Cell type	Carbohydrate	Enzymatic activities[a]		
		L-Fucose isomerase	Ribitol dehydrogenase	D-Ribulokinase
Parent strain W70	None	<0.004[b]	<0.01	<0.01
	D-Arabinose	<0.004	<0.01	<0.01
	L-Fucose	0.02	<0.01	<0.01
D-Arabinose-positive mutant *fucC6*	None	0.02	0.03	<0.01
	D-Arabinose	0.03	1.65	0.2

[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, where indicated, with 0.5% carbohydrate.

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

When this constitutive mutant was grown on casein hydrolysate, the presence of D-arabinose resulted in the induction of both enzymes of the ribitol pathway (Table 2).

When cells lacking L-fucose isomerase activity were grown on casein hydrolysate, the presence of D-arabinose did not lead to induction of either RDH or DRK, but with the L-fucose isomerase-constitutive cells, capable of isomerizing D-arabinose to D-ribulose, the isomerization of D-arabinose resulted in the induction of both enzymes of the ribitol pathway. The phosphorylation of the D-ribulose by the DRK of the ribitol pathway could allow utilization of D-arabinose as a carbon and energy source by the pathway represented in Fig. 1, as was found in *A. aerogenes* (3).

Characterization of ribitol-negative mutants. The mutants we had isolated, which were unable to elicit D-ribulokinase activity but produced ribitol dehydrogenase when grown on casein hydrolysate in the presence of ribitol, demonstrated the requirement in *K. aerogenes* for functional D-ribulokinase activity to permit growth at the expense of ribitol. We hoped to obtain similar data for the RDH requirement by isolating additional mutants which would not grow on ribitol and by utilizing as the parent a strain which was L-fucose isomerase constitutive. Mutants which would not grow on ribitol were isolated from *fucC6*, a D-arabinose-positive mutant constitutive for L-fucose isomerase. Three classes of mutants were obtained (Table 3). One class, represented by *rbtK4*, produced RDH but not DRK when grown on casein hydrolysate supplemented with ribitol or D-arabinose. These appeared to be the result of DRK structural gene mutations. A second class, represented by *rbtD2*, did not produce RDH or DRK in response to ribitol, but produced DRK in response to D-arabinose. These mutants would appear to carry RDH structural gene

TABLE 3. *Ribitol dehydrogenase and* D-*ribulokinase activities of ribitol-negative mutants*

Mutant	Supplement	Enzyme activities[a]	
		Ribitol dehydrogenase	D-Ribulokinase
fucC6	Ribitol	2.5	0.7
	D-Arabinose	2.0	0.5
RDH$^-$ (*rbtD1*)	Ribitol	<0.01[b]	<0.01
	D-Arabinose	<0.01	0.4
DRK$^-$ (*rbtK4*)	Ribitol	1.5	0.06
	D-Arabinose	3	0.05
Rbt$^-$ (*rbt-21*)	Ribitol	<0.01	<0.01
	D-Arabinose	<0.01	<0.01

[a] Enzymatic activities, expressed as micromoles per milligram of protein per minute, were obtained from cell-free extracts derived from *fucC6*, or its derivatives, grown on 1% casein hydrolysate supplemented with 0.5% of the indicated carbohydrate.

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

mutations. The third class, Rbt$^-$, failed to produce either RDH or DRK in response to ribitol or D-arabinose, even though they were still L-fucose isomerase constitutive and were capable of converting D-arabinose to D-ribulose. This phenotype was consistent with a mutation in a control site, a polar nonsense mutation in an operator-proximal structural gene, a deletion of both enzymes sites, or two separate mutations in the structural genes for RDH and DRK. DRK$^+$ revertants of these Rbt$^-$s, isolated for their ability to utilize D-arabinose, were also RDH$^+$ revertants, eliminating both the possibility of deletion of both structural genes and of two separate mutations in the structural genes, since the former would not yield true DRK$^+$ revertants and the latter would yield DRK$^+$ RDH$^-$ revertants to growth on D-arabinose. These mutations (*rbt-21*, *rbt-22*) appear at this

time to be either polar nonsense mutations or mutations in a regulatory site.

Coordinate control of the ribitol enzymes. In *K. aerogenes*, mutants selected for growth on the rare pentitol, xylitol, were constitutive for RDH (Table 4). All such mutants isolated were also constitutive for DRK (Table 4) even though RDH-constitutive, DRK-negative mutants which we constructed grew on xylitol, indicating that DRK was not required for growth on this pentitol. RDH-constitutive mutants isolated for their ability to reduce tetrazolium when given ribitol in the presence of chloramphenicol (12) were also uniformly DRK constitutive, even though this method for selection of RDH-constitutive mutants was also functional in DRK-negative strains. This indicated that RDH and DRK were coordinately controlled, since DRK constitutivity was not required for selection of RDH-constitutive mutants, but was found in all such mutants isolated. Also consistent with the concept of coordinate control was the fact that RDH was produced when cells were grown on D-arabinose, even though this enzyme was not required for growth on this substrate. With the exception of mutants containing a structural gene alteration, no mutants have been found which are constitutive for only one of these enzymes, even though procedures selective for only one enzyme have been employed. In addition, no conditions were found where only one enzyme was induced, even though conditions under which only one was required have been used.

Inducer of the ribitol pathway. Our inability to induce DRK activity in cells lacking RDH activity, when the cells were grown on casein hydrolysate supplemented with ribitol, indicated that ribitol was not the apparent inducer of the ribitol catabolic pathway. Cells unable to synthesize RDH but which were constitutive for the fucose pathway (so that they could isomerase D-arabinose to D-ribulose) induced D-ribulokinase when grown on D-arabinose, indicating that D-ribulose or a result of subsequent steps was the apparent inducer of this pathway, since D-arabinose did not induce either enzyme in mutants lacking L-fucose isomerase activity. Our ability to induce RDH activity in D-ribulokinase-negative mutants grown on casein hydrolysate supplemented with either ribitol or D-arabinose showed that the inducer occurred prior to the D-ribulokinase-catalyzed reaction. These data indicated that D-ribulose was the intermediate compound responsible for induction of this pathway.

TABLE 4. *Ribitol dehydrogenase and D-ribulokinase activities in xylitol-positive isolates*

Cell type	Mutant[a]	Enzymatic activities[b]	
		Ribitol dehydrogenase	D-Ribulokinase
K. aerogenes strain, W70 parent		<0.006[c]	<0.01
Xylitol positive	*rbtC11*	5.1	0.40
	rbtC13	6.1	0.55
	rbtC14	10	0.97
	rbtC16	4.0	0.33

[a] The isolates are results of independent mutational events.

[b] The enzymatic activities, expressed as micromoles per milligram of protein per minute, were obtained from cell-free extracts grown on 1% casein hydrolysate.

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

Genetic determinants of growth on ribitol. The mutants listed in Tables 3 and 4 were used in a series of two- and three-point crosses to determine the genetic relationships among the mutations found. In Table 5, basic two-point crosses are presented which show that the RDH and DRK structural genes (*rbtD*, *rbtK*) and the *rbt-21* and *rbt-22* loci are closely linked to the *rbtC* site. Transductants were selected on ribitol or D-arabinose to yield $rbtD^+$ or $rbtK^+$ transductants, respectively, and tested for growth on xylitol to identify *rbtC* co-transductants.

Figure 2 shows the basic three-point cross which was used to order the markers involved. The donor contained a mutation to constitutive synthesis of RDH and DRK (*rbtC14*). The recipient contained two mutations, the first being the *rbtD2* mutation described previously. Subsequently, a DRK-negative mutation, *rbtK8*, was selected from this strain for its inability to grow on D-arabinose. It still maintained constitutive levels of L-fucose isomerase but failed to elicit RDH or DRK activity when grown on casein hydrolysate supplemented with ribitol or D-arabinose. In the cross, DRK-positive transductants capable of growth on D-arabinose were selected. These transductants arose from crossovers originating to the right of *rbtK*, as drawn in Fig. 2, and terminating somewhere to the left of *rbtK* in region A, B, or C. Those terminating in region A received the $rbtK^+$ genotype but not the *rbtC14* or $rbtD^+$ genotypes, whereas those terminating in region B or C also received the $rbtD^+$ or $rbtD^+$ and *rbtC14* genotypes, respectively, in addition to the $rbtk^+$ genotype and could, therefore, grow (in addition to D-arabinose) on ribitol or ribitol and xylitol, respectively. The alternate configurations with *rbtC* between *rbtK* and *rbtD* or to the

TABLE 5. *Two-point crosses in the ribitol region of K. aerogenes W70*

Donor	Recipient	Selected marker	No. scored	Unselected marker	Co-transduction frequency (%)
rbtC11	*rbtD2*	*rbtD*$^+$	2,037	*rbtC11*	92.8
rbtC13	*rbtD2*	*rbtD*$^+$	900	*rbtC13*	94.2
rbtC14	*rbtD2*	*rbtD*$^+$	1,216	*rbtC14*	98.5
rbtC15	*rbtD2*	*rbtD*$^+$	2,414	*rbtC15*	98.5
rbtC16	*rbtD2*	*rbtD*$^+$	1,311	*rbtC16*	99
rbtC13	*rbtD1*	*rbtD*$^+$	945	*rbtC13*	98
rbtC13	*rbtD3*	*rbtD*$^+$	604	*rbtC13*	99
rbtC13	*rbtK10*	*rbtK*$^+$	1,083	*rbtC13*	89.2
rbtC13	*rbtD2 rbtK12*	*rbtK*$^+$	900	*rbtC13*	92.8
		rbtK$^+$	900	*rbtD*$^+$	98.5
rbtC14	*rbtD2 rbtK8*	*rbtK*$^+$	1,237	*rbtC14*	95.2
		rbtK$^+$	1,237	*rbtD*$^+$	96.6
rbtC13	*rbt-21*	*rbt*$^+$	558	*rbtC13*	55.6
rbtC13	*rbt-22*	*rbt*$^+$	694	*rbtC13*	94.6

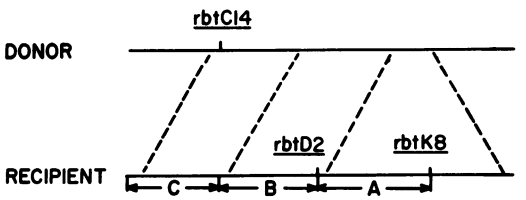

FIG. 2. *Diagram of the crosses which gave the data in Table 6, where crossovers originating to the right of the selected marker rbtK*$^+$ *terminate to the left of rbtK in region A, B, or C.*

right of *rbtK* should both result in a large number of the *rbtK*$^+$ *rbtD2 rbtC14* genotypes, in the cross illustrated, if *rbtK*$^+$ were the selected marker. The latter class would be identified as D-arabinose-positive, ribitol-negative, xylitol-negative transductants which were constitutive for DRK.

In two independent crosses of this type, we used separate *rbtC* and *rbtK* mutations and selected for the DRK$^+$ (*rbtK*$^+$) phenotype (Table 6). No *rbtK*$^+$-*rbtD2-rbtC14* (or *rbtC13*) recombinant types were found in either case, as would be expected if *rbtC* were between *rbtD* and *rbtK* or if *rbtK* were between *rbtC* and *rbtD*. However, recombinants of the type described above for crossovers terminating in regions A, B, and C for the map order with *rbtD* central to the *rbtK* and *rbtC* loci were readily found in both cases. The gene order was, therefore, assumed to be as indicated in Fig. 3. The mutations resulting in the Rbt$^-$ phenotype, the double negatives from Table 3, have not been included in the map because the loci involved cannot be identified by three-point crosses with markers available at this time.

DISCUSSION

We have been able to identify in *K. aerogenes* strain W70 two enzymes, RDH and DRK, which are induced in response to D-ribulose, an apparent intermediate of a specific ribitol catabolic pathway. A similar pathway is found in *A. aerogenes* strain PRL-R3 (22) composed of two enzymes similar in terms of induction and activity. Our decision to shift studies of *A. aerogenes* PRL-R3 to *K. aerogenes* W70 was influenced by the discovery of a transduction system mediated by phage PW52, which was reported by MacPhee et al. (13) and the discovery that *K. aerogenes* W70 was similar to *A. aerogenes* strain PRL-R3 with respect to its degradation of naturally occurring C_5 compounds, and in its adaptation to growth on the uncommon C_5 sugars and alcohols. The lone dissimilarity in this area was the inability of *K. aerogenes* to degrade those C_5 compounds which in *A. aerogenes* appear to be catabolized by a pathway involving L-xylulokinase. L-Xylulokinase activity is demonstrable in *A. aerogenes*, but we have been unable to demonstrate it in *K. aerogenes*. The procedures worked out for utilizing *A. aerogenes* for selection and isolation of mutants were, therefore, modified to work in *K. aerogenes* W70.

The excellent background work in *A. aerogenes* on ribitol catabolism allowed us to develop similar information for *K. aerogenes* W70 on induction of the enzymes involved, the apparent inducer, and mode of adaptation to growth on the rare sugars D-arabinose and xylitol.

The enzymes RDH and DRK are coordinately controlled, since mutants selected for constitutive synthesis of RDH by their ability to grow with xylitol as a carbon and energy source, or for the ability of starved cells to reduce tetrazolium dyes in the presence of ribitol, are constitutive

TABLE 6. *Results of the three-point crosses*[a]

Donor	Recipient: *rbtD2* and	No. of transductants				
		Region A			Region B	Region C
		Total DRK$^+$ scored	DRK$^+$ RDH$^-$ Rbt^l	DRK$^+$ RDH$^-$ Rbt^c	DRK$^+$ RDH$^+$ Rbt^l	DRK$^+$ RDH$^+$ Rbt^c
rbtC13	*rbtK12*	900	14	0	52	834
rbtC14	*rbtK8*	1,237	45	0	15	1,177

[a] The crosses, with donors and recipients both containing the same uracil-negative, L-ribulokinase-negative, L-fucose isomerase constitutive (*fucC6*), and L-fucose-negative mutations, were carried out as described in the text. The second cross is represented in Fig. 2; the first cross is analogous. DRK$^+$ is the phenotype for *rbtK*$^+$; DRK$^-$ is the phenotype for *rbtK8* and *rbtK12;* RDH$^+$ and RDH$^-$ are the phenotypes for *rbtD*$^+$ and *rbtD2*, respectively. Rbt^l corresponds to *rbtC*$^+$ and Rbt^c corresponds to *rbtC13* and *rbtC14*.

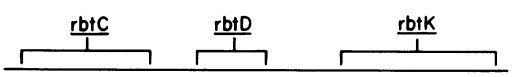

FIG. 3. *rbt region in Klebsiella aerogenes strain W70, where the brackets define the limits of all such mutants tested on a relative scale.*

for DRK, even though we can demonstrate the effectiveness of the selections in mutants lacking a functional DRK. In addition, L-fucose isomerase-constitutive cells, when grown on D-arabinose, produce high levels of RDH and DRK even though RDH is not involved in growth on that substrate. We have found that L-fucose isomerase in *K. aerogenes*, as in *A. aerogenes*, converts D-arabinose to D-ribulose. This leads to induction of the enzymes of the ribitol pathway. Mutants capable of producing D-ribulose from D-arabinose, but unable to synthesize RDH, still produce DRK in response to the D-ribulose, indicating that reduction to the pentitol is not required for induction of DRK. Mutants lacking DRK activity, although unable to grow on D-arabinose alone, produce, when grown on casein hydrolysate supplemented with D-arabinose, high levels of RDH, indicating that the DRK is not required for production of the inducer from the D-ribulose formed. The role of D-ribulose as an inducer of these enzymes is consistent with our inability to find mutants which produce DRK but not RDH when grown on casein hydrolysate supplemented with ribitol, even though mutants which appear to induce DRK but not RDH are found if D-arabinose rather than ribitol is offered as substrate.

In addition to helping us identify the inducer of the ribitol pathway, growth on D-arabinose shows that *K. aerogenes*, like *A. aerogenes*, mutates preferentially to constitutive synthesis of the L-fucose catabolic enzymes (in particular L-fucose isomerase) rather than to a modification that allows induction of these enzymes in cells grown on D-arabinose, as found in *Escherichia coli* (10). The latter alternative, however, can also be found in this organism. In a similar manner, growth on the unusual pentitol, xylitol, is the result of a mutation to constitutive synthesis of the ribitol catabolic enzymes. In this case RDH converts xylitol to D-xylulose. The D-xylulose produced can be phosphorylated by specific D-xylulokinases. This will be considered in greater detail in a subsequent paper.

Our understanding of growth on D-arabinose and xylitol allowed us to map the sites for phenotypes which we had surmised to represent lesions in structural and regulatory sites of the ribitol pathway enzymes. Prior to setting up the crosses reported, we were obliged to construct a mutant which would allow us to score the various phenotypes on semisolid media. We, therefore, created a mutant with the following lesions: (i) uracil requiring, to allow us to identify contaminants as such if they arose; (ii) L-ribulokinase negative, to eliminate conversion by this enzyme of D-ribulose to D-ribulose-5-phosphate as reported in *A. aerogenes* (10); (iii) L-fucose isomerase constitutive, to allow isomerization of D-arabinose to D-ribulose and subsequent growth potential on D-arabinose through the DRK as illustrated in Fig. 1; (iv) L-fucose negative, to prevent shunting of D-ribulose through the L-fuculokinase and fucose aldolase, as reported in *E. coli* (9), in those mutants lacking a functional DRK.

In this basic mutant we were able to set up two- and three-point crosses with mutants with the phenotypes listed in Tables 1 and 2, i.e., mutants which constitutively synthesized RDH and DRK or failed to produce one or both of these enzymes under conditions where these enzymes should have been induced. The lesions were all closely linked genetically, as shown in the two-point crosses, and three-point crosses

yielded the map order indicated in Fig. 3.

The map order is consistent with our concept of an operon, and the induction of the enzymes is consistent with the functioning of an operon. We have not examined the direction of transcription or the possibility of positive or negative control. Thus, although we have no data for this system which are inconsistent with the concept of an operon, we do not have sufficient evidence to report it as an operon. We have recently found that there appear to be at least two genetically distinguishable control sites, as defined by mutations to constitutive synthesis of RDH and DRK, but we cannot yet define them in terms of operator and regulator functions. This aspect of the work will be considered in a subsequent paper.

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