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Close Genetic Linkage of the Determinants of the Ribitol and D-Arabitol Catabolic Pathways in *Klebsiella aerogenes*

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Klebsiella aerogenes strain W70 has separate inducible pathways for the degradation of the pentitols ribitol and D-arabitol. These pathways are closely linked genetically as determined by transduction with phage PW52. There are two regulatory sites for the ribitol catabolic pathway as defined by loci for mutations to constitutive synthesis of ribitol dehydrogenase and D-ribulokinase, *rbtB* and *rbtC*. The two control sites are separated by a site represented by the *dalB22* mutation. This mutation deprives the cell of the ability to induce synthesis of D-arabitol dehydrogenase and D-xylulokinase activities. Two additional regulatory mutations for the D-arabitol pathway, *dalC31* and *dalC37*, map to the opposite side of *rbtB13* relative to *dalB22*. The order of the genetic sites thus far determined for this region is *dalK-dalD-dalC31*, *dalC37-rbtB13-dalB22-rbtC14-rbtD-rbtK*, where *dalK* and *dalD* represent structural genes for the kinase and dehydrogenase of the D-arabitol pathway, respectively, and *rbtK* and *rbtD* represent the corresponding genes for the ribitol pathway. The two mutations that lead to constitutive synthesis of the D-arabitol-induced enzymes, *dalC31* and *dalC37*, have different phenotypes with regard to their response to xylitol. The growth of *dalC31* is inhibited by xylitol, but the toxicity can be reduced by increasing the levels of ribitol dehydrogenase either by induction with ribitol or by selection of a ribitol dehydrogenase-constitutive mutation.

We have previously described two distinct pathways in *Klebsiella aerogenes* strain W70 for the catabolism of the two naturally occurring pentitols, ribitol and D-arabitol (2, 3). The ribitol catabolic pathway is comprised of at least two enzymes, ribitol dehydrogenase (EC 1.1.1.56), which oxidizes ribitol to the 2-keto sugar D-ribulose, and D-ribulokinase (EC 2.7.1.47), which phosphorylates the D-ribulose. In a similar manner the D-arabitol catabolic pathway is also comprised of at least two enzymes: a dehydrogenase, D-arabitol dehydrogenase (EC 1.1.1.11), which oxidizes D-arabitol to D-xylulose, and a pentulokinase, D-xylulokinase (EC 2.7.1.17), which phosphorylates the D-xylulose produced. The two enzymes of each pathway are coordinately controlled and induced when the cells are grown on the respective substrates. Genetically each pathway has been described as containing at least two structural genes, coding for the dehydrogenase and kinase of that pathway, and a regulatory site. Three genetic components of each pathway are closely linked, with the dehydrogenase structural gene central to the regulatory site and the kinase structural gene in each case.

This paper presents data indicating two regulatory sites for each pathway and describes the close genetic linkage between these two pathways as determined by transduction.

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MATERIALS AND METHODS

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. The mutants used are those described in previous papers (2, 3), plus additional mutants derived in this laboratory by the methods described in those papers.

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The procedures for maintenance of cultures, growth and assay of phage PW52, and transductions were described previously (2). Growth of cells for preparation and assay of cell-free extracts were as previously described for ribitol catabolic enzymes (2) and for D-arabitol catabolic enzymes (3).

Enzymatic assays. D-Xylose isomerase (EC 5.3.1.5), D-ribulokinase (EC 2.7.1.47), and D-xylulokinase (EC 2.7.1.17) (1), and D-arabitol dehydrogenase (EC 1.1.1.11) and ribitol dehydrogenase (9) were assayed as described previously.

Chemicals. D-Xylulose (6) and D-ribulose (2) were prepared in this laboratory by previously reported procedures. D-Ribulose was isolated as the *O*-nitrophenolhydrazone derivative (4) and regenerated as D-ribulose for use (7). Additional chemicals were obtained from commercial sources as indicated previously (2, 3).

RESULTS

Genetic linkage of the pentitol dehydrogenase genes. Figure 1 shows the type of crosses which demonstrated the high co-transduction frequency for the genes coding for the enzymes ribitol dehydrogenase and D-arabitol dehydrogenase. In these crosses, we used as recipients mutants which were ribitol dehydrogenase negative (*rbtD2*) (2) and D-arabitol dehydrogenase negative (*dalD1* or *dalD2*) (3), and a donor which was constitutive for the ribitol pathway (*rbtB13*) (2). (*rbtB13*, previously *rbtC13*, is separated from the *rbtC* mutation by genetic analysis presented in this paper.)

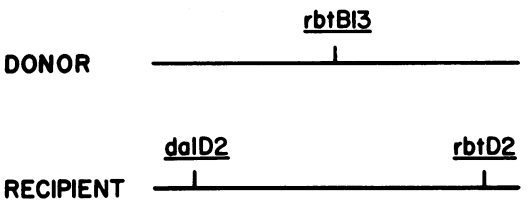

FIG. 1. *Diagram of the crosses (data presented in Table 1) which demonstrate close genetic linkage of the D-arabitol dehydrogenase structural gene (dalD2), the ribitol dehydrogenase structural gene (rbtD2), and a regulatory site for the ribitol pathway (rbtB13).*

When we selected *rbtD*$^+$ transductants on ribitol and tested them for co-transduction of the *dalD*$^+$ gene, we found that over 90% of these *rbtD*$^+$ transductants were *dalD*$^+$ (Table 1). Similar data were found when we selected *dalD*$^+$ transductants and tested for co-transduction of the *rbtD*$^+$ gene. Essentially all of the *dalD*$^+$ *rbtD*$^+$ co-transductants were *rbtB13* (constitutive) as judged by their ability to grow with xylitol as the sole carbon and energy source (Table 1). Where *rbtD*$^+$ was the selected marker, it indicated that the *rbtB13* gene was located between the *dalD* and *rbtD* genes. The same pattern of recombination was found when an *rbtC14* mutant was used as donor (data not shown).

It has been shown that the (*rbtB*) *rbtC*, *rbtD*, and *rbtK* sites are closely linked and in the order (*rbtB*) *rbtC-rbtD-rbtK* (2). These crosses gave us the expanded order *dalD*-(*rbtB*) *rbtC-rbtD-rbtK*.

Similar crosses (Fig. 2) with one *rbtD2*, *dal*$^-$ recipient (*dal-21*) gave the same phenotypes as the *dalD* mutant, indicating that it too was to the left of *rbtB13* as drawn in Fig. 1. The *dal*$^-$ mutants are those which have simultaneously lost the ability to induce either D-arabitol dehydrogenase or D-xylulokinase activities and are unable to grow at the expense of D-arabitol. The second mutant, *dalB22*, gave the same *dal*$^+$ *rbtD*$^+$ *rbtB13* recombinants in a similar cross, but included in the transductants were *dal*$^+$ *rbtD*$^+$, *rbtB*$^+$ recombinants when *dal*$^+$ or *rbtD*$^+$ was the selected marker, and absent were the *rbtD*$^+$, *rbtB13 dalB22* recombinants when *rbtD*$^+$ was the selected marker. This indicated that the *dalB22* site was to the right of *rbtB13* as drawn in Fig. 2.

Demonstration of two control sites for the ribitol pathway. The location of the *dalB22* site was examined further with the third cross

TABLE 1. *Results of three-point crosses demonstrating the linkage of the D-arabitol dehydrogenase structural gene, ribitol dehydrogenase structural gene, and the rbtB*$^+$ *site*[a]

Pertinent markers		Selected marker	No. of transductants			
Donor	Recipient		DalD$^+$ Rbt^c	DalD$^+$Rbt$^+$	DalD$^-$ Rbt$^+$	DalD$^-$ Rbt^c
rbtB13	*rbtD2 dalD1*	*rbtD*$^+$	594	0	13	16
rbtB13	*rbtD2 dalD2*	*rbtD*$^+$	2,510	3	40	91

[a] Both donor and recipient contained the following mutations: Uracil negative-*M1*, L-ribulokinase negative-*4*, L-fucose isomerase constitutive (*fucC6*), L-fucose negative (*fuc-1*), D-xylose negative-*7A*, mannitol negative-*1*. The phenotypes DalD$^+$ and DalD$^-$ correspond to the genotypes *dal*$^+$ and *dalD1* and *dalD2*. Rbt$^+$ and Rbt^c correspond to *rbtB*$^+$ and *rbtB13*, respectively.

(Table 2). The first two crosses, *rbtB13* × *rbtD2 dal-21* and *rbtB13* × *rbtD2 dalB22*, demonstrated that the *dal-21* site was to the left of *rbtB13* and the *dalB22* site was to the right of *rbtB13*. The third cross demonstrated that the *dalB22* site was to the left of *rbtC14*, since the *rbtD*$^+$ transductants included *dalB22 rbtC14* and *dalB*$^+$ *rbtC*$^+$ genotypes but none of the *rbtD*$^+$ *dalB*$^+$ *rbtC*$^+$ genotypes expected if the *dalB22* site were between *rbtD2* and *rbtC14* or to the right of *rbtC14* as drawn. It should be noted that *rbtC14* (or *rbtB13*) *dalB22* mutants do not grow on xylitol because they are unable to induce D-xylulokinase. Therefore, all *dalB22* phenotypes were grown and assayed for constitutive synthesis of ribitol dehydrogenase.

The second and third crosses in Table 2 demonstrate that there are two distinct regulatory sites for the control of the synthesis of ribitol dehydrogenase and D-ribulokinase as defined by mutations to constitutive synthesis of these enzymes. The sites for *rbtB13* and *rbtC14* are separate, and the designation *rbtB* is given to the *rbtB13* site (previously *rbtC13*). The order of these sites as indicated by these data is *rbtB13-dalB22-rbtC14-rbtD-rbtK*. The two constitutive mutations, *rbtB13* and *rbtC14*, are indistinguishable physiologically at this time. Both result in constitutive synthesis of ribitol dehydrogenase and D-ribulokinase, and both permit growth on the rare pentitol xylitol.

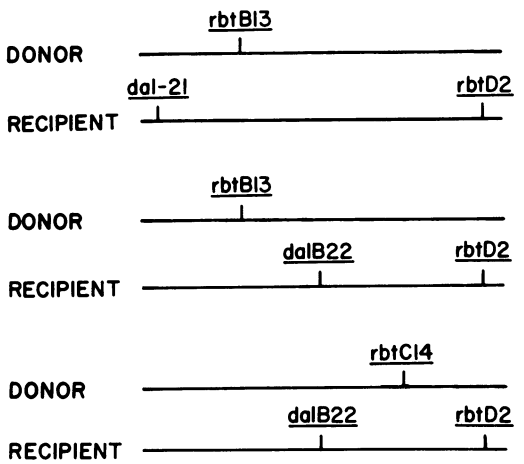

FIG. 2. *Diagrams of the crosses (data presented in Table 2) which demonstrate two regulatory sites (rbtB13, rbtC14) for the ribitol pathway and identify the location of one regulatory site for the D-arabitol pathway (dalB22).*

Demonstration of two sites controlling synthesis of D-arabitol dehydrogenase and D-xylulokinase. The first two crosses in Table 2 were particularly important because they demonstrated the existence of two sites which, when mutated, lead to the inability to produce either D-arabitol dehydrogenase or D-xylulokinase in response to D-arabitol. It was not clear whether both of these mutants were regulatory in nature. The mutation *dalB22* (previously designated *dal-22*) appeared to be regulatory in nature based on the following observations. As reported previously (3), *dalK*$^+$ strains are able to grow utilizing xylitol as the sole carbon and energy source if they are constitutive for ribitol dehydrogenase (*rbtC* or *rbtB*), which catalyzes the oxidation of xylitol to D-xylulose, an intermediate in D-arabitol degradation. Under these conditions, both D-arabitol dehydrogenase and D-xylulokinase are induced. Mutants constitutive for ribitol dehydrogenase (*rbtB13* and *rbtC14*) are unable to induce D-arabitol dehydrogenase or D-xylulokinase in response to xylitol if they have a *dalB22* mutation. In addition, a revertant of *dalB22* was obtained which was constitutive for both the dehydrogenase and kinase of the D-arabitol pathway. Such a mutant was not found for the *dal-21* site, and ribitol dehydrogenase-constitutive mutants elicited D-arabitol dehydrogenase and D-xylulokinase activity in

TABLE 2. *Three-point crosses demonstrating two control sites for the ribitol pathway*[a]

Donor	Recipient	Selected marker	No. of transductants			
			Dal$^+$ Rbt^c	Dal$^+$ Rbt$^+$	Dal$^-$ Rbt$^+$	Dal$^-$ Rbt^c
rbtB13	*rbtD2 dal-21*	*rbtD*$^+$	1,098	0	36	2
rbtB13	*rbtD2 dal-22*	*rbtD*$^+$	548	5	8	0
rbtC14	*rbtD2 dal-22*	*rbtD*$^+$	948	0	4	43

[a] Both donor and recipient contained the following mutations: Uracil negative-*M1*, L-ribulokinase negative-*4*, L-fucose isomerase constitutive (*L-fucC6*), L-fucose negative (*fuc-1*), D-xylose negative-*7A*, mannitol negative-*1*. The phenotypic designations Dal$^+$ and Rbt$^+$ indicate inducible for the D-arabitol and ribitol pathways (*dal*$^+$, *rbtB*$^+$, and *rbtC*$^+$), Dal$^-$ indicates not inducible for the D-arabitol pathway (*dal-21*, *dal-22*), and Rbt^c corresponds to constitutive for the ribitol pathway (*rbtB13* or *rbtC14*).

response to xylitol in the growth medium even when the *dal-21* mutation was present.

Table 3 shows data obtained from transductions using a donor constitutive for the ribitol pathway (*rbtB13*, Table 1) and also constitutive for the D-arabitol pathway (*dalC31* or *dal C37*). The recipient was either a ribitol dehydrogenase-negative (*rbtD2*, *rbtB*$^+$) or a D-arabitol dehydrogenase (*dalD2*, *rbtB*$^+$) transductant from Table 1. Selection for transductants positive for either dehydrogenase (*rbtD*$^+$ or *dalD*$^+$) demonstrated linkage of the D-arabitol-constitutive mutations to that dehydrogenase. The recipient and donor both contained the mannitol-1 mutation, which allowed us to score the (*dalC*) mutants by their ability to grow at the expense of D-mannitol (3).

When *dalD*$^+$ was the selected marker, we would expect to find recombinant types that resulted from crossovers originating to the left of *dalD2* in region A and terminating in regions B, C, and D + E to yield *dalC*$^+$ *rbtB*$^+$, *dalC*$^-$, *rbtB*$^+$, and *dalC*$^-$ *rbtB13* recombinant types but few, if any, *dalC*$^+$ *rbtB13* recombinants, if the order was as indicated with the *dalC31* and *dalC37* mutations between the *dalD2* and *rbtB13* sites. The *dalC*$^+$ *rbtB13* recombinant type should be frequent if the *dalC* mutations were to the left of the *dalD2* or to the right of *rbtB13*, but in fact none were found (Table 3) even though reasonable numbers of recombinants were found for the classes expected for the gene order indicated. Similar crosses with the *rbtD*$^+$ as the selected marker should give us recombinants, as shown in Fig. 3, from crossovers which originated to the right of *rbtD2* in region E and terminated in regions A + B, C, or D to yield *rbtB13 dalC31* (or *rbtB13 dalC37*) recombinants (regions A + B), *rbtB13 dalC*$^+$ recombinants (region C), and *rbtB*$^+$ *dalC*$^+$ recombinants (region D), but not *rbtB*$^+$ *dalC31* recombinants, if our ordering of mutations is correct. Again the expected recombinant types were found, confirming the order *dalD-dalC31 dalC37-rbtB13*. This gives us an expanded gene order *dalD-dalC-rbtB-dalB-rbtC-rbtD-rbtK* and indicates two distinct sites affecting synthesis of the enzymes of the D-arabitol catabolic pathway, one represented by the constitutive mutants *dalC31* and *dalC37* and the second by the *dalB22* mutation which leads to simultaneous loss of both enzymatic activities.

The *dalC31* and *dalC37* mutations were mapped relative to the *dalD* and *dalK* mutations, in a previous paper (3), to give the order *dalK-dalD-dalC*. The total order for this region as determined to date is given in Fig. 4. The brackets indicate the limits of all mutations of

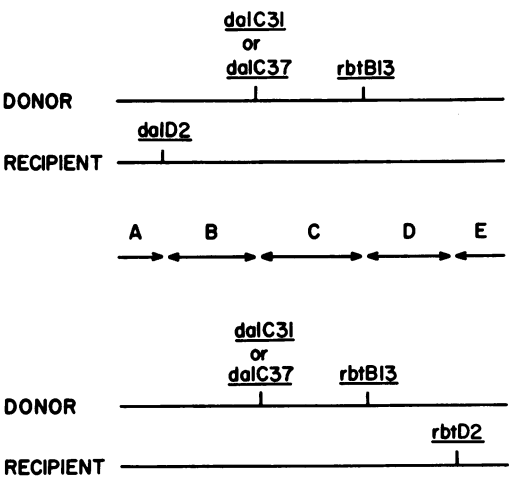

FIG. 3. *Diagrams of the crosses (data presented in Table 3) which identify a second regulatory site for the D-arabitol catabolic pathway represented by dalC31 and dalC37.*

TABLE 3. *Results of the three-point crosses used to map the D-arabitol pathway constitutive mutations dalC31 and dalC37*[a]

Pertinent markers		Selected marker	No. of transductants			
Donor	Recipient		Dal$^+$ Rbt^C	Dal^C Rbt$^+$	Dal$^+$ Rbt^C	Dal$^+$ Rbt$^+$
rbtB13 dalC31	*dalD2*	*dalD*$^+$	805	31	0	20
	rbtD2	*rbtD*$^+$	966	0	4	7
rbtB13 dalC37	*dalD2*	*dalD*$^+$	781	37	0	29
	rbtD2	*rbtD*$^+$	755	0	37	64

[a] Both donor and recipient contained the following mutations: Uracil negative-*M1*, L-ribulokinase negative-*4*, L-fucose isomerase constitutive (*fucC6*), L-fucose negative (*fuc-1*), D-xylose negative-*7A*, mannitol negative-*1*. The phentypes Dal$^+$ and Rbt$^+$ are inducible for D-arabitol and ribitol pathways, respectively (*dalC*$^+$, *rbtC*$^+$, and *rbtB*$^+$). Dal^C and Rbt^C are the constitutive phenotypes for D-arabitol and ribitol pathways, respectively (*dalC31*, *dalC37*, and *rbtB13*).

that class on a scale relative to the co-transduction frequencies.

Separation of dalC31 and dalC37 into two physiological groups. Although *dalC31* and *dalC37*, the two *dalC* mutants mapped in Table 3, appear to map at a single site, they are representative of two distinct physiologically identifiable groups. Both types of *dalC* mutants were isolated by their ability to grow with D-mannitol as the sole carbon and energy source, and both had essentially the same growth rate on minimal salts with glucose, ribitol, D-arabitol, or mannitol as the sole carbon and energy source. The growth rates on xylitol were different; 0.048 generations per h for *dalC31* and 0.12 generations per h for *dalC37*. The parent strain ARX 3-2 (*rbtB13*) had a generation time on xylitol of 0.15 generations per h, close to the growth rate for the *dalC37* mutant.

When we examined the levels of the ribitol and D-arabitol dehydrogenase in these two mutants, we found no significant difference in cells grown on casein hydrolysate, ribitol, D-arabitol, or on xylitol. When cells were grown on casein hydrolysate supplemented with xylitol, however, a dramatic difference was found (Table 4).

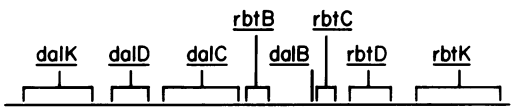

FIG. 4. *The rbt-dal region in Klebsiella aerogenes strain W70.*

The parent strain ARX 3-2, a *rbtD*$^+$ *dalD*$^+$ *rbtB13* transduction from the cross in Table 1, and the *dalC37* mutant maintained their high constitutive levels of ribitol dehydrogenase. The mutant *dalC31*, however, behaved quite differently. Although this mutant was able to maintain high constitutive levels of both dehydrogenases on casein hydrolysate, or on 1% xylitol alone, it produced barely detectable levels of these enzymes on casein hydrolysate supplemented with 1% xylitol (Table 4).

Since growth on xylitol was dependent on ribitol dehydrogenase constitutivity (*rbtB13*) and the difference in *dalC31* and *dalC37* was a difference in response to xylitol in the growth media, we isolated a *dalC31 rbtB*$^+$ (D-arabitol dehydrogenase-constitutive, ribitol dehydrogenase-inducible) mutant from the cross in Table 3. This mutant was able to grow on casein hydrolysate (1 generation per h) but unable to grow on casein hydrolysate supplemented with 1% xylitol. Since this strain was not induced or constitutive for ribitol dehydrogenase, it could not catalyze the oxidation of xylitol to D-xylulose, indicating that xylitol rather than a product of its catabolism was responsible for the growth inhibition. Selection of a ribitol dehydrogenase-constitutive mutant partially relieved the inhibition and permitted some growth (0.05 generations per h), as did induction of ribitol dehydrogenase by inclusion of ribitol in the growth medium.

Thus, although both the *dalC37* and *dalC31* mutations result in constitutive synthesis of

TABLE 4. *D-Arabitol dehydrogenase and ribitol dehydrogenase activities*[a] *of dalC31 and dalC37 mutants*

Strain	Supplements	Activity	
		D-Arabitol dehydrogenase	Ribitol dehydrogenase
K. aerogenes W70	CH	<0.02[b]	<0.02
	CH + xylitol	<0.02	<0.02
(ARX 3-2) Parent strain for *dalC*$^+$ mutants	CH	<0.03	5.1
	CH + xylitol	1.1	4.7
	Xylitol	2.2	4.0
dalC37	CH	1.9	5.0
	CH + xylitol	1.2	2.2
	Xylitol	1.6	3.1
dalC31	CH	2.3	3.0
	CH + xylitol	0.06	0.03
	Xylitol	1.1	2.4

[a] Activity, expressed as micromoles per milligram of protein per minute, in cell-free extracts of cells grown on minimal salts supplemented with 1% casein hydrolysate and/or 1% xylitol.

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

both enzymes of the D-arabitol catabolic pathway, the *dalC31* mutation also results in an inhibition of growth by xylitol. High levels of ribitol dehydrogenase (induced or constitutive) can catalyze the oxidation of xylitol and partially overcome the growth inhibition.

DISCUSSION

Our interest in the catabolism of ribitol and D-arabitol was heavily influenced by our interest in the evolution of enzyme systems. We were interested in finding a system which might have evolved by duplication and modification of one pathway to yield a second independent pathway.

We chose to examine the pentitol catabolic pathways because (i) the two naturally pentitols are degraded by a limited number of organisms; and (ii) in *Aerobacter aerogenes* strain PRL-R3 at least, the mechanisms for degradation are similar. In each case, the pentitol is oxidized to a 2-keto sugar by a distinct pentitol dehydrogenase, and then phosphorylated to a pentulose-5-phosphate by a kinase specific for the pentulose produced. The dehydrogenase and kinase of each pathway are coordinately controlled.

We chose to examine *K. aerogenes* strain W70 rather than *A. aerogenes* strain PRL-R3 because of a readily available genetic exchange system for the former which was unavailable at the time for the latter. In two previous papers we were able to identify a specific catabolic pathway for each pentitol in *K. aerogenes*. We demonstrated dehydrogenase and kinase activities for each pathway, examined the regulation of each pathway enough to determine the apparent inducer in each case, D-arabitol for its pathway and the intermediate D-ribulose for the ribitol pathway, and determined a genetic order for the loci involved. We were not surprised to find in each case that the two structural genes linked closely to the regulatory site, with the dehydrogenase structural gene located central to the kinase structural gene and the regulatory site. In addition, the co-transduction frequencies were comparable for the two pathways.

Each pathway now appears to have two distinct regulatory sites, a finding consistent with our present concept of operons, but we have been unable to assign regulator or operator functions, in spite of obvious spatial considerations, because of our inability to generate diploids.

The most striking genetic discovery to date has been the close proximity of these two pathways. The entire region between the two kinases is over 80% co-transducible, and our co-transduction frequencies for available markers indicate that there may be a continuous segment of deoxyribonucleic acid coding exclusively for the pentitol catabolic-related regulatory and catalytic functions.

That portion of our genetic map between the dehydrogenases appears to contain two regulatory sites for each pathway. The two sites for ribitol pathway regulation, represented by *rbtB13* and *rbtC14*, are identified as sites for constitutive mutations.

One of the "sites" for D-arabitol regulation, represented by *dalC31* and *dalC37*, may, in fact, represent two sites. There are distinct physiological differences between these two mutants, both of which are constitutive for the D-arabitol catabolic enzymes. The most notable difference is their response to xylitol. A marked reduction in both constitutive dehydrogenase activities, found at high xylitol concentrations (1.0%) in the *dalC31* mutant, was not found in the *dalC37* mutant, and a *dalC31* rbt^+ mutant which we constructed appeared unable to grow on casein hydrolysate in the presence of xylitol, whereas a *dalC37*, rbt^+ mutant could.

A second site apparently involved in regulation of the D-arabitol enzymes is represented by the *dalB22* mutation. As a result of this mutation, the cells are apparently unable to induce either the dehydrogenase or kinase of that pathway in response to D-arabitol or to xylitol under conditions where they would otherwise have done so. This site is separated genetically from the *dalC31*, *dalC37* site, in addition to the sites for dehydrogenase and kinase, by the *rbtB13* site.

Our present understanding of regulatory mechanisms would indicate that the two regulatory sites for each pathway should represent a regulator and operator site in each case. Although this can only be surmised from the location of the sites, we might reasonably assume that transcription should originate near the regulatory sites adjacent to the dehydrogenase genes (*rbtC14*, *dalC31*, and *dalC37*). This would indicate translation to the "left" in the case of the D-arabitol pathway and to the "right" for the ribitol pathway and necessitate transcription of one strand in one case and the alternate strand in the other. Demonstration of regulator and operator functions and the direction(s) of transcription should be most interesting considering the relative positions of the regulatory sites which we surmise to be "regulator" in nature (*rbtB13*, *dalB22*).

ACKNOWLEDGMENT

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