Regulation of Pentitol Metabolism by *Aerobacter aerogenes*  

II. Induction of the Ribitol Pathway

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The incubation of *Aerobacter aerogenes* PRL-R3 with ribitol resulted in the induction of ribitol dehydrogenase and D-ribulokinase, coordinately controlled enzymes of the pathway of ribitol catabolism. A dehydrogenase-negative mutant was unable to induce D-ribulokinase activity following incubation with ribitol. Similar experiments using a kinase-negative mutant resulted in normal induction of ribitol dehydrogenase, as compared to the wild-type PRL-R3 strain. Constitutive or induced cells for L-fucose isomerase were capable of catalyzing the isomerization of D-arabinose to D-ribulose. In contrast to the experiments using ribitol as the substrate, the isomerization of D-arabinose resulted in the induction of D-ribulokinase with dehydrogenase-negative cells. These data indicated that D-ribulose, rather than ribitol, acts as the inducer of the enzymes for ribitol degradation.

*Aerobacter aerogenes* PRL-R3 catabolizes ribitol by a pyridine nucleotide-linked oxidation to yield the 2-ketopentose, D-ribulose. D-Ribulose is then phosphorylated to form D-ribulose-5-phosphate (12). The enzymes catalyzing these two reactions appear to be coordinately controlled, since mutants which were constitutive for ribitol dehydrogenase were also constitutive for D-ribulokinase. Mutants possessing the ability to utilize the aldopentose, D-arabinose, as the sole carbon and energy source for growth can be selected. Such mutants degrade D-arabinose by isomerization to D-ribulose followed by phosphorylation to D-ribulose-5-phosphate. This isomerization is catalyzed by L-fucose isomerase, an enzyme normally induced by L-fucose. The mutational event permitting degradation of D-arabinose causes this non-specific L-fucose isomerase (2) to be constitutively synthesized. With respect to its physical and immunological properties (6), the D-ribulokinase present in D-arabinose-grown cells was shown to be identical to the kinase purified from ribitol-grown cells, and extracts of D-arabinose-grown cells were proportionally more active for the coordinately controlled ribitol dehydrogenase (7). Since D-arabinose itself does not act as an inducer for kinase or dehydrogenase (8), the catabolism must result in the synthesis of the actual inducer for these two enzymes of ribitol degradation. To identify the inducer of the enzymes of ribitol catabolism, mutants which were unable to utilize ribitol as a substrate for growth were isolated, and these mutants were examined for their ability to induce the enzymes of the ribitol pathway when incubated with ribitol or D-arabinose as substrates.

**MATERIALS AND METHODS**

**Isolation of mutants.** The parent strain of *A. aerogenes* used in these experiments was a uracil-requiring auxotroph. This strain was obtained by treating the PRL-R3 strain with ultraviolet (UV) light and was isolated by replica plating. Strains 176 and 179 were obtained by treatment of the uracil-requiring auxotroph with *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (Aldrich Chemical Co.), followed by penicillin enrichment and replica plating. These strains were isolated because of their inability to grow with ribitol as the sole carbon source. Strain 181 was isolated in a similar manner after treatment of the uracil-requiring auxotroph with UV light. Strains AC1 and 181-A were isolated from the PRL-R3 and 181 strains, respectively. They were selected for their ability to grow when D-arabinose was the sole carbon and energy source.

Replica-plating experiments utilized replica-plating discs which consisted of velveteen glued to pile. To prepare these discs, velveteen was cut into circular discs measuring 25 mm in diameter, and these discs were glued to similar discs cut from #V22-90 tile (Velcro Corp., New York, N.Y.) so that the pile was exposed. These velveteen-Velcro discs were stored in petri dishes and were sterilized by autoclaving. To hold the discs in place during replica-plating experiments, strips of Velcro #65 hook were glued to cover the end of a conventional replica base. To fasten a
disc to the base, the hook end of the base was pressed on the Velcro pile side of a disc. After use, the disc was removed from the base and another disc was fastened onto the base in a similar manner.

Growth and induction experiments utilized a basal salts medium (7) supplemented with 0.005% uracil and 0.5% of each of the indicated carbon and energy sources. Cultural conditions were described previously (7). During induction experiments, cultures were diluted into fresh medium to maintain a cell mass of less than 1 mg (dry weight) per ml.

**Enzymes assays.** Methods for preparation of cell-free extracts and protein and enzyme assays were described previously (7). Ribitol dehydrogenase and D-ribulokinase were assayed spectrophotometrically employing a model 2000 recording spectrophotometer (Gilford Instrument Laboratories, Inc.). Isomerase assays were carried out by use of the cysteine-carbazole test (3) for measuring the amount of ketopentose synthesized per a given time period (1). One unit of ribitol dehydrogenase or D-ribulokinase was capable of causing an absorbancy change of 1.0 per minute at 340 mp in a reaction volume of 0.15 ml. One unit of isomerase in 2.0 ml catalyzed the formation of 1 mumole of ketopentose per hour at 37 C.

**Reagents.** Ribitol, D-arabinose, L-fucose, and vitamin-free casein hydrolysate were purchased from the Nutritional Biochemicals Corp., and D-ribulose-o-nitrophenylhydrazone was obtained from Calbiochem. D-Ribulose was prepared by the oxidation of ribitol using purified ribitol dehydrogenase (9).

**RESULTS**

Three mutants were isolated which appeared to be unable to utilize ribitol as a growth substrate. Preliminary experiments showed that mutant strain 176 was unable to synthesize an active D-ribulokinase. Strains 179 and 181 were isolated as ribitol dehydrogenase-deficient mutants, but, upon closer examination, strain 179 was found to synthesize a low level of dehydrogenase activity and to be capable of slow growth with ribitol as the substrate. These three mutant strains, as well as wild-type PRL-R3 strain, were cultured in a casein hydrolysate medium, and the cells were then transferred to a ribitol-casein hydrolysate medium. The results of this induction experiment are shown in Fig. 1. The spectrophotometric assays employed in these experiments lost their accuracy at specific activities below 1.0, which represent minute differences in slope relative to the control cuvettes measuring adenosine triphosphatase or nicotinamide adenine dinucleotide (NADH) oxidase activity. As seen in Fig. 1, dehydrogenase-negative strain 181 was incapable of inducing kinase activity during incubation with ribitol. With the 179 leaky dehydrogenase strain, kinase synthesis was delayed, when compared to the wild-type PRL-R3 strain. These data suggest that D-ribulose, the intermediate metabolite of the pathway, rather than ribitol itself, may function as the actual inducer of the two coordinately controlled enzymes.

To determine if incubation of strain 181 with D-ribulose would result in the induction of kinase activity, cells were incubated for 5 hr in a minimal medium supplemented with various concentrations of D-ribulose. The results of this experiment are shown in Table 1. In the case of the 181 mutant, higher activity for D-ribulokinase was detected when the D-ribulose concentration was increased from 0.25 to 0.76%. Since this mutant was incapable of reducing D-ribulose to ribitol and the combination of 0.5% ribitol and 0.25% D-ribulose did not result in greater induction of D-ribulokinase, the data shown in Table 1 support the concept that D-ribulose, rather than ribitol, functions as the true inducer of these enzymes.

Cells of *A. aerogenes* grown or induced with the methyl pentose, L-fucose, were capable of isomerizing D-arabinose to D-ribulose. This activity is believed to be catalyzed by the L-fucose isomerase (2). Experiments in which the organism was cultured in an L-fucose-salts medium and then was transferred to a D-arabinose-salts medium provided a means for the synthesis of D-ribulose within the cell without the permeability problems encountered when D-ribulose itself was utilized as the substrate. Figure 2 illustrates the increase in kinase and dehydrogenase activities following such transfer experiments. The induction of kinase with dehydrogenase-negative strain 181 and leaky strain 179 supported the hypothesis that ribulose, rather than ribitol, functions as the ac-
tual inducer. As mentioned previously, D-arabinose itself did not function as an inducer for these enzymes, and controls in which cells were transferred from a casein-hydrolysate medium to a D-arabinose medium resulted in no detectable kinase or dehydrogenase activity during the course of the experiment.

When *A. aerogenes* is incubated continuously in a D-arabinose-salts medium, mutants capable of utilizing D-arabinose as the sole carbon and energy source for growth can be selected. Such mutants have been shown to be constitutive for D-arabinose isomerase (L-fucose isomerase) activity (2). An isomerase-constitutive derivative of the PRL-R3 strain which was isolated by this method was termed AC₄, and, following growth in a casein hydrolysate medium, cell-free extracts possessed 68 units per mg of protein of D-arabinose isomerization activity. A constitutive derivative of strain 181 was also isolated, and the isomerase activity, determined after growth on casein hydrolysate, was 16.1 units per mg of protein. Both of these constitutive strains were cultured in a casein hydrolysate medium and then were transferred to a minimal medium supplemented with 0.5% D-arabinose. Samples of the cultures were removed at intervals, and cell-free extracts were prepared and assayed for kinase and dehydrogenase activities. The results of this experiment are plotted in Fig. 3. Throughout the course of the experiment, ribitol dehydrogenase activity was not detected with strain 181. Since this strain was incapable of reducing ribulose to ribitol, the induction of kinase activity gave further evidence that D-ribulose, rather than ribitol, functions as the true inducer. It is of interest that the slopes of these induction curves appear to be proportional to the constitutive isomerase activity of the organisms. Thus, for strain AC₄, the ratio of the induction slopes of dehydrogenase and kinase, when compared to the slope of kinase induction for strain 181, is 4.2:1, and the ratio of the constitutive isomerase activities of these organisms is also 4.2:1. Although a large number of mutants should be tested to confirm this ratio, the data suggest that the slope of induction of dehydrogenase and kinase is dependent upon the maximal rate of isomerization of D-arabinose to D-ribulose in the constitutive mutants.

**DISCUSSION**

In the preceding study, we found that, in *A. aerogenes* strain PRL-R3, a single inducer, ribitol,
induced the synthesis of the two coordinately controlled enzymes, ribitol dehydrogenase and D-ribulokinase. However, when the 181 mutant of \textit{A. aerogenes} was incubated with ribitol, kinase was not induced. Thus we concluded that ribitol was not the actual inducer and could not give rise to the actual inducer in this dehydrogenase-negative strain, and we reasoned that D-ribulose must be the actual inducer. It also seems likely that, with kinase-negative strain 176, D-ribulose, rather than ribitol or D-ribulose-5-phosphate, functions as the inducer, since strain 176 synthesized dehydrogenase when incubated with ribitol and D-ribulose-5-phosphate was an intermediate in the degradation of other carbohydrates (such as D-ribose).

In a number of catabolic pathways, the first substrate does not function as the inducer. Magasanik and his co-workers showed that the first two enzymes in the histidine degradative pathway of \textit{A. aerogenes} are coordinately induced by the product of the first enzyme reaction, urocanic acid (5, 11), rather than L-histidine. In the tryptophan degradative pathway of \textit{Pseudomonas fluorescens}, the product of the second degradative enzyme, L-kyurenine, induces all of the enzymes required to convert L-tryptophan to anthranilic acid and L-alanine (10). Koch, Hayashi, and Lin (4) reported that \(\alpha\)-glycerol phosphate, rather than glycerol, is the true inducer of glycerol kinase. The pathway of ribitol degradation in \textit{A. aerogenes} appears to be another example of induction by an intermediate metabolite.

Although D-ribulose is metabolized through D-ribulose as an intermediate, it cannot be assumed that natural selection for the induction of D-ribulokinase by D-ribulose has occurred merely to facilitate the degradation of D-ribulose. \textit{A. aerogenes} apparently does not possess the genetic capability of synthesizing an isomerase specific for the D-ribulose pathway, and the isomerization of D-ribulose to D-ribulose occurs only with mutants constitutive for L-fucose isomerase or with cells previously induced for this enzyme with L-fucose (2).

Since D-ribulose is the inducer of the coordinately controlled kinase and dehydrogenase, the rate of synthesis of these enzymes and, thus, the rate of catabolism of ribitol are dependent upon the internal pool of D-ribulose, which, in turn, is dependent upon the relative rates of activity of both enzymes. Thus, the relative degree of induction can be dependent upon such factors as the availability of adequate electron acceptors, and the cell may possess additional control for the synthesis of these enzymes.

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**LITERATURE CITED**

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