Short Communication

Evolution of D-Arabinose, L-Xylose and L-Ribose Utilization in *Mycobacterium smegmatis*: Mutants with a Novel Enzyme "Pentose Reductase"

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Mycobacterium smegmatis was noted for its ability to produce D-ribose isomerase whose existence was first reported by the authors.¹⁾ Also the organism seemed to be unusual among microorganisms in that the inducer-specificity of pentose isomerases was relatively broad.²⁾ By comparing the structures of inducers of D-lyxose isomerase, it was postulated that the β -D-lyxose should be the inducer of the enzyme.³⁾

The metabolism of various pentoses in *Mycobacterium smegmatis* seemed to proceed by way of the corresponding ketopentoses. Namely, D-xylose, L-arabinose, D-lyxose and D-ribose isomerase activities were found in the cells grown on D-xylose, L-arabinose, D-lyxose and D-ribose, respectively.²⁾ It seemed likely that L-lyxose was metabolized by D-ribose isomerase which is active on and able to be induced by L-lyxose.⁴⁾ Of eight pentoses, D-arabinose, L-xylose and L-ribose were not able to serve as carbon sources for growth. D-Arabinose isomerase was not found in the cells incubated with D-arabinose or L-fucose.²⁾ The inability to utilize L-fucose by *M. smegmatis* implies that this organism is deficient in L-fucose (D-arabinose) isomerase.

During studies on growth of *M. smegmatis* on various pentoses, we discovered that the

organism began to grow on D-arabinose, L-xylose and L-ribose after long incubation in a medium containing these pentoses as a carbon source. In this report we describe some experiments on the evolution of pathways for growth on these novel pentoses.

Growth was observed after one day of incubation when D-xylose, L-arabinose, D-lyxose, L-lyxose, D-arabitol, L-arabitol, xylitol, ribitol, D-glucose, D-mannose, D-fructose, L-sorbose, L-rhamnose, sorbitol or mannitol were used as the sole source of carbon. After a long time lag of eight to ten days, growth of cells appeared in the medium containing D-arabinose, L-xylose, D-ribose, L-ribose or D-galactose. Other sugars tested, L-glucose, L-galactose, L-mannose, D-fucose, L-fucose, dulcitol, lactose and sucrose, did not serve as growth substrates for *M. smegmatis*.

The mutants that grew on D-arabinose, L-xylose, L-ribose, D-ribose and D-galactose were selected after long incubation and maintained on the slants of Ogawa medium consisting of 1.0 g of KH_2PO_4 , 1.0 g of sodium glutamate, 200 g of chicken egg, 10 g of glycerol, 0.12 g of brilliant green and 110 g of water. The mutant strain selected for growth on D-arabinose (D-Ara⁺ mutant), that was transferred to Ogawa medium several times, was inoculated in the mineral salts medium²⁾ with various sugars. As shown in Fig. 1, D-Ara⁺ mutant could grow on D-arabinose without long time lag. Long time lag also disappeared when the D-Ara⁺ mutant grew on not only D-arabinose but also L-xylose and L-ribose. However, the mutant still grew on D-ribose and D-galactose after a long lag, and could not grow on other sugars which the parent strain could not utilize as a growth substrate.

The same growth experiments were carried out on various mutants: L-Xyl⁺, L-Rib⁺, D-Rib⁺ and D-Gal⁺ mutants. The lengths of time lag of these mutants for growth on various sugars were determined and are shown in Table I. L-Xyl⁺ and L-Rib⁺ mutants grew on D-arabinose, L-xylose and L-ribose without long lag, but still a long time lag was observed in the medium with D-ribose or D-galactose. In

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FIG. 1. Growth Curves Obtained for D-Ara⁺ *M. smegmatis* on Various Sugars.

Symbols: (○), D-arabinose; (△), L-xylose; (□), L-ribose; (●), D-ribose; (▲), D-galactose.

the D-Rib⁺ and D-Gal⁺ mutants, a long lag disappeared only when the mutants grew on D-ribose and D-galactose, respectively, and still needed long incubation to grow on D-arabinose, L-xylose and L-ribose.

It seemed possible that in D-Ara⁺, L-Xyl⁺ and L-Rib⁺ mutants a similar enzyme system was working on the metabolic pathways of D-arabinose, L-xylose and L-ribose.

We tried to find a novel metabolic pathway for utilization of D-arabinose in D-Ara⁺ mutant. D-Arabinose uptake activity was

assayed by the use of D-[U-¹⁴C]-arabinose as reported previously for *Klebsiella aerogenes*.⁵⁾ The activity in the cells of D-Ara⁺ mutant grown on D-arabinose or various sugars was determined at about 4 μmol per g of dry cells per min and that was about five to ten times larger than that in the parent strain grown on various sugars. The uptake activity was inhibited by the simultaneous addition of various non-radioactive carbohydrates. These results suggested that D-arabinose was incorporated by the nonspecific permease system.

D-Arabinose isomerase activity in the cells of D-Ara⁺ mutant grown on D-arabinose was assayed at various pH with or without Mn²⁺, Mg²⁺ or Co²⁺. Under any reaction conditions no activity of D-arabinose isomerase was detected in the cells. Nor was D-arabinose isomerase detected in the cells incubated with L-fucose which was a substrate and inducer of D-arabinose (L-fucose) isomerase in *Klebsiella aerogenes*.^{6,7)} D-Arabinose kinase and D-arabinose dehydrogenase activities were not found in the cells grown on D-arabinose, either.

The crude extract of D-Ara⁺ mutant grown on D-arabinose was found to contain D-

TABLE I. TIME LAG OF GROWTH OF MUTANT STRAINS ON VARIOUS SUGARS

Carbon source	Time lag of growth on various sugars ^a					
	Parent	D-Ara ⁺	L-Xyl ⁺	L-Rib ⁺	D-Rib ⁺	D-Gal ⁺
D-Arabinose	7-8	1	1	1	8-9	7-9
L-Arabinose	1	1	1	1	1	1
D-Lyxose	1	1	1	1	1	1
L-Lyxose	1	1	1	1	1	1
D-Ribose	7-9	7-8	9-10	8-10	1	9-10
L-Ribose	7-8	1	1	1	5-6	5-6
D-Xylose	1	1	1	1	1	1
L-Xylose	8-9	1	1	1	9-10	7-9
D-Arabitol	1	1	1	1	1	1
L-Arabitol	1	1	1	1	1	1
Ribitol	1	1	1	1	1	1
Xylitol	1	1	1	1	1	1
D-Glucose	1	1	1	1	1	1
D-Galactose	8-10	8-9	10-12	9-10	10-12	2
D-Mannose	1	1	1	1	1	1

^a Days required before any increase in growth could be measured.

arabinose reductase activity that required NADPH₂ as a coenzyme. Specific activity was determined as 3.0×10^{-3} units per mg of protein. The optimum pH of the reductase was found at 6.0. Substrate specificities for various sugars were determined and are shown in Table II. The reductase found in the cells did not seemed to be specific for D-arabinose, and a higher activity was found against D-lyxose and L-ribose. Almost the same amount of the reductase was detected in the cells of D-Ara⁺ mutant grown on other sugars. Thus the reductase seemed to be constitutively produced in D-Ara⁺ mutant. The reaction product of D-arabinose reductase was determined with crude extract and D-[U-¹⁴C]-arabinose as the substrate. As shown in Fig. 2, D-arabitol was detected when NADPH₂ was added to the reaction mixture. A little reductase activity also was determined in the cells of the parent strain. However, the amount of the activity was less than five percent of that of D-Ara⁺ mutant.

Studies on experimental evolution of bacteria have been extensively done during recent years⁸⁻¹³⁾ and reviewed by Hegeman and Rosenberg¹⁴⁾ and Wu.¹⁵⁾ There are two different pathways for D-arabinose metabolism by mutants selected for growth on D-arabinose. In D-Ara⁺ *Klebsiella aerogenes*, the mutation allowing D-arabinose utilization is associated with the constitutive synthesis of an

FIG. 2. Radioactivity of Products from D-[U-¹⁴C]-Arabinose by Enzyme Reaction with Crude Pentose Reductase.

After enzyme reaction, the radioactive products were chromatographed in the solvent, methylethyl ketone, acetate, saturated borate solution (8:1:1), on Toyo No. 50 paper. Then the paper was cut into 0.5-cm strips, and the radioactivity was determined with a liquid scintillation spectrometer. Also shown are cold D-arabinose and D-arabitol.

A, complete reaction mixture; B, without NADPH₂; C, boiled enzyme used.

L-fucose isomerase acting on L-fucose and D-arabinose.^{6,7,16)} On the other hand, in D-Ara⁺ *Escherichia coli*, D-arabinose induces the synthesis of three enzymes normally associated with L-fucose catabolism, L-fucose isomerase, L-fuculokinase and L-fuculose-1-phosphate aldolase, and all three dual substrate specificity.^{17,18)} In the parent strain and D-Ara⁺ mutant of *Mycobacterium smegmatis*, L-fucose was not utilized as a growth substrate and no activity of L-fucose isomerase was found in the cells. In D-Ara⁺ *Klebsiella aerogenes* PRL-R3⁶⁾ and *K. aerogenes* M-7,⁵⁾ D-arabinose was incorporated with cells by L-fucose (D-arabinose) permease which was specific for D-arabinose and L-fucose. In D-Ara⁺ *M. smegmatis*, however, D-arabinose seemed to be incorporated by non-specific aldose permease.

With the demonstration of constitutive

TABLE II. SUBSTRATE-SPECIFICITY OF CRUDE PENTOSE REDUCTASE FROM D-ARA⁺ *Mycobacterium smegmatis*

Pentose	Activity (%) ^a	Hexose	Activity (%) ^a
D-Lyxose	100	D-Mannose	10
L-Ribose	61	D-Galactose	8
L-Xylose	29	D-Glucose	6
D-Xylose	27	L-Glucose	6
D-Ribose	25	L-Mannose	4
D-Arabinose	14	L-Galactose	1
L-Arabinose	10	L-Fucose	4
L-Lyxose	9	L-Rhamnose	4

^a D-Lyxose, 100%.

production of D-arabinose reductase in the cells of D-Ara⁺ mutant, the major growth characteristics on D-arabinose become interpretable. In *M. smegmatis*, four pentitols were good substrates for growth.²⁾ If the novel pentoses were reduced to certain pentitols by the reductase produced constitutively, the strain should be able to utilize the novel pentoses as growth substrates.

As shown in the growth experiments, it was assumed that D-arabinose, L-xylose and L-ribose should be metabolized by a similar metabolic system in D-Ara⁺, L-Xyl⁺ and L-Rib⁺ mutants. From the fact that the non-specific pentose reductase was found in not only D-Ara⁺ mutant but also L-Xyl⁺ and L-Rib⁺ mutants, constitutively, the metabolic pathway of these novel pentoses should be postulated as follows. D-Arabinose, L-xylose and L-ribose were incorporated by the non-specific aldose permease and reduced by the reductase to D-arabitol, xylitol and ribitol, respectively.

L-Ribose was utilized by D-Ara⁺, L-Xyl⁺ and L-Rib⁺ mutants as a growth substrate. These strains were the first organisms that were found capable of growth on the unnatural pentose, L-ribose, as a sole source of carbon.

Our present study demonstrates another strategy how a new catabolic pathway of novel pentoses can be constructed by a derepressing enzyme which may fortuitously act on novel pentoses. However, the origin of the derepressing enzyme, non-specific pentose reductase that has a high activity against unnatural pentoses, D-lyxose and L-ribose, is not clear.

So we are now in the process of purifying the reductase to determine the properties of the enzyme and confirming the origin of the novel enzyme.

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