Selective Inhibition of *Klebsiella aerogenes* Growth on Pentoses by Pentitols

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Selective inhibition of growth by pentitols was observed when *Klebsiella aerogenes* M-7 which could not utilize pentitols was grown on pentoses. D-Arabitol inhibited the growth on D-arabinose as a sole carbon source, but had no effect on the growth on L-arabinose, D-xylose, and D-ribose. Similarly, L-arabitol inhibited the growth on D-arabinose and L-arabinose, ribitol inhibited the growth on D-arabinose and L-arabinose, and xylitol inhibited the growth on D-xylose. From the following reasons, we postulated that the selective growth inhibition by pentitols was due to the competitive inhibition of pentose isomerase reaction in the cell by pentitols. (i) D-Arabinose transport activity was not inhibited by pentitols. (ii) Induction of D-arabinose and L-arabinose isomerases was not inhibited by D- and L-arabitol, respectively. (iii) The specificity of growth inhibition by pentitols was the same as that of competitive inhibition of pentose isomerases by pentitols.

Klebsiella aerogenes PRL-R3 has been reported to be capable of growth on seven of the eight aldopentoses and all of the four pentitols (8). Pentitols were metabolized to the corresponding 2-ketopentoses by respective pentitol dehydrogenases. However, *K. aerogenes* M-7, which was isolated from seawater in our laboratory, was capable of utilizing five of the eight pentoses (growth on D-xylose, D-arabinose, L-arabinose, D-ribose, and D-lyxose; no growth on L-xylose; growth not determined for L-lyxose and L-ribose) as sole carbon sources (5). In contrast to *K. aerogenes* PRL-R3 and *K. aerogenes* 1033 (4), our strain, *K. aerogenes* M-7, was unable to utilize characteristically any of the four pentitols as growth substrate.

The inability to utilize pentitols by *K. aerogenes* M-7 implies that this strain is deficient in pentitol dehydrogenases. The finding of principal interest is that pentitols are nonmetabolite analogs of pentose for this strain. Pentitols are known to be competitive inhibitors for pentose isomerases, e.g., xylitol for D-xylose isomerase (12), L-arabitol and ribitol for L-arabinose isomerase (6, 9, 10), and D-arabitol for D-arabinose isomerase (7).

During studies on the growth rate of *K. aerogenes* M-7 on various compounds, it was found that D-arabitol inhibited the growth of the organism on D-arabinose. However, the growth on

D-glucose was not inhibited by D-arabitol. Thus, it was of interest to examine the mechanism of selective inhibition of growth by D-arabitol which was not utilized by the strain.

In this report, we describe some experiments on the selective inhibition of *K. aerogenes* M-7 growth on pentoses by pentitols.

MATERIALS AND METHODS

Bacterial strain and media. *K. aerogenes* M-7 was used throughout these studies. The mineral medium contained 0.24% KH_2PO_4 , 0.56% K_2HPO_4 , 0.26% $(\text{NH}_4)_2\text{SO}_4$, 0.01% MgCl_2 , and 0.005% MnCl_2 . Medium A had an identical composition, except that 0.01% yeast extract was added. Basal medium contained 0.5% each of yeast extract, peptone, and NaCl (pH 7.0). Addition of pentose and pentitol to the medium is specified in the text.

Growth experiments. Growth experiments were performed in exponential-phase cultures. Inoculum was made in 5 ml of medium A in a test tube (16.5-mm diameter) and incubated at 30°C with reciprocal shaking. Growth was followed by measuring the absorbance directly at 600 nm on a Hitachi model 101 spectrophotometer with the aid of a test-tube holder. Absorbance at 600 nm of 1.0 was equivalent to 0.356 mg (dry weight) of cells per ml of medium.

Induction of pentose isomerases. *K. aerogenes* M-7 was grown on the basal medium for 18 to 20 h. Cells were harvested and washed with chilled 0.05 M glycine-NaOH buffer (pH 9.3) and transferred to fresh medium A containing 0.2% D-arabinose or L-arabinose in a total volume of 5.0 ml. Cells were incubated at 35°C for 3 h with constant shaking to induce the pentose isomerases. Within the induction period, only little growth was observed. After 3 h of incubation,

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cells were collected by centrifugation and suspended in 0.05 M glycine-NaOH buffer (pH 9.3), containing 1 mM each of MnCl_2 and mercaptoethanol. The level of the enzyme induced in the cells were measured after treatment of the cells with toluene by the procedure of Dobrogosz and DeMoss (2) and of Chakravorty (1). The toluenized cells were used in the following assay system for the isomerase. The assay for isomerases was carried out as described previously (6, 7). One unit of enzyme is defined as the amount which produces 1 μmol of ribulose per min under the standard conditions.

D-Arabinose uptake activity. *K. aerogenes* M-7 was grown on medium A supplemented with 0.5% various pentoses. Cells were washed and added to the mineral medium supplemented with 0.1% glycerol, 500 μg of chloramphenicol per ml, and a suitable concentration of radioactive D-arabinose. The total volume of the reaction mixture was 5 ml. After mixtures were maintained at 35°C in a shaking water bath, samples (1 ml) were removed at specified times and vacuum filtered onto Sartorius membrane filters (pore size, 0.45 μm). The filters were immediately washed with 1 ml of the mineral medium, air dried, and mounted on cupped aluminum planchets for detection of radioactivity. Experimental results are expressed as micro-moles of D-arabinose accumulated per gram (dry weight) of cells per 10 min. Radioactivity was determined by using a standard Geiger-counting technique with a gas-flow counter (Aloka model PDC-R8282). D-[U- ^{14}C]arabinose (Amersham/Searle) was used without further purification.

RESULTS

Effect of pentitols on the growth on pentoses. Growth experiments were performed with medium A containing 0.2% pentose and 2% pentitol in various combinations. D-Arabitol inhibited the bacterial growth on D-arabinose, and little growth was observed even after 48 h of shaking (Table 1). Ribitol also inhibited the growth, but to a lesser degree than did D-arabitol. Inhibition of growth was observed with L-arabitol at 24 h, but growth was recovered to the normal state after 48 h. Xylitol did not inhibit the growth. Further studies were carried out on the effect of D-arabitol on growth on D-arabinose with varying amounts of D-arabitol and a con-

TABLE 1. Effect of pentitols on the growth on D-arabinose^a

Culturing time (h)	Bacterial growth ($A_{600\text{ nm}}$) ^b				
	Without pentitol	With D-arabitol	With L-arabitol	With Ribitol	With xylitol
0	0.22	0.27	0.18	0.22	0.18
24	1.29	0.24	0.46	0.41	1.20
48	1.20	0.36	1.20	0.78	1.20

^a Bacteria were inoculated in medium A with 0.2% D-arabinose and 2% pentitol in each test tube.

^b $A_{600\text{ nm}}$, Absorbance at 600 nm.

stant amount of D-arabinose (0.2%). The concentration of D-arabitol was varied from 0.1 to 1.0%, and growth was recorded hourly for a 13-h period. The growth rate on D-arabinose decreased with increasing concentrations of D-arabitol (Fig. 1). No change was observed in length of lag phase and final cell yield.

Similar growth experiments were performed with L-arabinose and D-xylose as carbon sources. Growth on L-arabinose was inhibited by L-arabitol and ribitol, but was recovered to the normal state after 48 h. D-Arabitol and xylitol did not cause any noticeable delay of the growth on L-arabinose. Growth on D-xylose was inhibited by xylitol; the other three pentitols, D-arabitol, L-arabitol, and ribitol, were completely inert.

In contrast to the above observations, growth on D-ribose was not affected by the presence of any of four pentitols. For growth on D-glucose, no inhibitory action was observed with any of the four pentitols.

It was indicated that there may be a distinct relationship between the structure of the inhibitory pentitol and that of the pentose used as growth substrates.

Constants for pentitol inhibition of growth on D-arabinose. We determined graphically the initial growth velocity on D-arabinose at an early stage of growth by use of Fig. 1, and kinetic studies were carried out. A plot of $\log[(v/v_i) - 1]$ versus $\log[\text{D-arabitol}]$, which is a Hill plot (3), yields a straight line (Fig. 2). In the equation, v and v_i are initial growth velocities on D-arabinose at logarithmic phase without and with D-arabitol, respectively. The concentration of D-arabitol which exhibited $\log[(v/v_i) - 1] = 0$ was estimated from the line to be 0.018 M. Hence, this value exhibited the concentration of D-arabitol that gives 50% inhibition of growth rate when the concentration of

FIG. 1. Inhibition of the cellular growth on D-arabinose with various amounts of D-arabitol. Numbers on the right indicate the concentration of D-arabitol (%) added in each test tube.

D-arabinose in the medium is 0.2% (0.013 M). This value was constant for each analog, and may be compared to a K_i value for enzyme inhibition. This inhibition constant was denoted by ϕ . The constants, ϕ , for L-arabitol and ribitol were determined graphically by the same manner to be 0.063 M and 0.060 M, respectively.

The slope of the line (Fig. 2), n , was calculated to be 1.02. For three pentitols, n values were found, unchanged, to be near 1.0. D-Arabinose isomerase from this strain was inhibited competitively by pentitols (7), and n values for all pentitols were calculated to be 1.0. In an enzyme inhibition reaction, n value indicates the number of inhibitor molecules that bind to an enzyme molecule. On the other hand, in growth inhibition, it is not clear what the n value means. However, these results suggested that the mechanisms of growth inhibition by these pentitols were identical to each other.

The inhibition constants, ϕ and n , are summarized in Table 2.

Effect of pentitols on the D-arabinose uptake activity. D-Arabinose uptake activities were induced in the cells grown on D-arabinose (205 $\mu\text{mol/g}$ per 10 min), D-lyxose (101 $\mu\text{mol/g}$ per 10 min), and L-fucose (150 $\mu\text{mol/g}$ per 10 min). Negligible activity (2 to 7 $\mu\text{mol/g}$ per 10 min) was found in the cells grown on L-arabinose, D-xylose, D-ribose, and D-glucose. Apparent K_m value for D-arabinose (concentration of radioactive D-arabinose in the reaction medium) was calculated to be 5×10^{-4} M.

The effect of various compounds, including four pentitols, was determined (Table 3). The addition of cold D-arabinose, D-ribose, and L-fucose inhibited the incorporation of radioactive D-arabinose. However, four pentitols and other pentoses tested did not inhibit the uptake activity, but stimulated it by about 5 to 20%.

Effect of pentitols on the pentose isom-

FIG. 2. Linear relationship between the growth rate of D-arabinose and the concentration of D-arabitol.

TABLE 2. Constants for pentitol inhibition of the growth on D-arabinose^a

Pentitol	n	ϕ (M)
D-Arabitol	1.02	0.018
L-Arabitol	1.14	0.063
Ribitol	0.99	0.060

^a Growth experiments were carried out with 0.2% (0.013 M) D-arabinose. The constants, ϕ , exhibited the concentration of pentitols giving 50% inhibition of growth rate when the concentration of D-arabinose in the medium was 0.013 M. The constants, n , indicated the apparent Hill coefficient.

TABLE 3. Effect of various compounds on D-arabinose uptake activity^a

Addition (20 mM)	D-Arabinose uptake activity (%)
None	100
D-Arabinose	11.4
L-Arabinose	123
D-Xylose	120
L-Xylose	114
D-Lyxose	103
D-Ribose	68.3
L-Fucose	24.8
D-Arabitol	105
L-Arabitol	127
Xylitol	124
Ribitol	124

^a Experimental conditions are described in the text. The concentration of D-[U-¹⁴C]arabinose was 1 mM, and 20 mM of unlabeled compounds was added simultaneously in the reaction medium.

erase induction. In *K. aerogenes* M-7, D-arabinose and L-arabinose are metabolized by D- and L-arabinose isomerases induced by these pentoses. Two percent of D-arabitol was sufficient to inhibit the growth on D-arabinose (Table 1). However, addition of 2% D-arabitol to the induction medium did not inhibit but slightly stimulated D-arabinose isomerase induction (Table 4). Similarly, the induction of L-arabinose isomerase was not inhibited by L-arabitol. The addition of D-glucose to the induction medium repressed the induction of both isomerases.

DISCUSSION

The inhibition of cellular growth by an analog of a normal metabolite is usually the result of several events. One type of inhibition is a direct consequence of the blocking of a transport process by the analog. The second type of inhibition occurs when the analog incorporated into the cell inhibits some biochemical reaction, preventing the cell from performing a vital function.

From the following reasons, we postulated that the selective growth inhibition by pentitols

TABLE 4. *Effect of pentitols on the isomerase induction^a*

Inducer (0.2%)	Addition (2%)	Isomerase induced (mU/mg of cell)
For D-arabinose isomerase ^b		
None	None	0.3
D-Arabinose	None	9.0
D-Arabinose	D-Arabitol	10.1
D-Arabinose	D-Glucose	0.0
For L-arabinose isomerase ^c		
None	None	0.5
L-Arabinose	None	14.5
L-Arabinose	L-Arabitol	17.4
L-Arabinose	D-Glucose	0.2

^a Conditions for the enzyme induction are described in the text.

^b For induction of D-arabinose isomerase, 16.8 mg (dry weight) of cells was used in each test tube.

^c For induction of L-arabinose isomerase, 17.6 mg (dry weight) of cells was used.

was due to the competitive inhibition of pentose isomerase reaction by pentitols.

(i) D-Arabinose transport activity was not inhibited by pentitols. As shown in Table 3, no inhibitory effect of pentitols on the uptake activity of D-arabinose was observed.

(ii) Induction of D-arabinose and L-arabinose isomerases was not inhibited by D- and L-arabitol, respectively. The results in induction experiments showed that D-arabitol and L-arabitol in sufficient concentration for growth inhibition had no inhibitory effect on induction of D- and L-arabinose isomerases, respectively.

(iii) The specificity of growth inhibition by pentitols was the same as that of competitive inhibition of pentose isomerases by pentitols. The pathways of pentose and pentitol metabolism by *K. aerogenes* PRL-R3 was elucidated by Mortlock and Wood (8). D-Arabinose, L-arabinose, and D-xylose were metabolized to the corresponding 2-ketopentose by respective isomerases, but D-ribose was first phosphorylated by a specific kinase to ribose-5-phosphate which was then isomerized to ribulose-5-phosphate. The same pathways of pentose metabolism were suggested in *K. aerogenes* M-7 (5).

If the activity of an intracellular pentose isomerase was blocked by pentitol, the incorporated pentose would not be expected to be utilized for the proliferation of bacteria. Growth on D-ribose was not inhibited by any of the four pentitols. Insensitivity of the growth on D-ribose to pentitols can be explained by the lack of D-ribose

TABLE 5. *Relationship between growth inhibition and inhibition of pentose isomerases by pentitols*

Carbon source and pentose isomerase	Growth inhibition ^a and <i>K_i</i> values (mM)			
	D-Arabitol	L-Arabitol	Ribitol	Xylitol
D-Arabinose	++ ^a	+	+	—
D-Arabinose isomerase ^b	1.3	2.9	2.2	10.0
L-Arabinose	—	+	+	—
L-Arabinose isomerase ^b	160	2.2	1.6	27.0
D-Xylose	—	—	—	++
D-Xylose isomerase ^c	130	146	— ^d	2.7

^a Symbols: ++, little growth was observed after 48 h; +, inhibition was observed after 24 h but growth was recovered after 48 h; —, no inhibition was observed.

^b Crystalline enzyme from *K. aerogenes* M-7 (6, 7).

^c Crystalline enzyme from *Lactobacillus brevis* (12).

^d Little inhibition was observed.

isomerase in its metabolic pathway.

In Table 5, the relationship between the degree of growth inhibition and *K_i* values for pentitols of pentose isomerases is summarized. When a *K_i* value for a pentitol of a certain pentose isomerase was smaller than about 3 mM, the growth of the organism on the pentose was inhibited by the pentitol. On the other hand, pentitols which have larger *K_i* values than 3 mM were found not to affect the growth. The specificity of both growth inhibition and competitive inhibition of pentose isomerases by pentitols was clearly identical.

Inhibition constants, ϕ obtained on three pentitols for growth on D-arabinose correlated well with the inhibition constants for D-arabinose isomerase. *K_i* values of D-arabinose isomerase from *K. aerogenes* M-7 are 1.3, 2.2, and 2.9 mM (7), and ϕ values are 18, 60, and 63 mM for D-arabitol, ribitol, and L-arabitol, respectively. The relation between two inhibition constants also provides the fundamental base of our assumption on the growth inhibition.

We postulate the mechanism of growth inhibition on D-arabinose by pentitols as follows. D-Arabinose is incorporated into the cell by D-arabinose permease and then isomerized to D-ribulose by D-arabinose isomerase induced by D-arabinose. Pentitols do not interfere with the permease and the inducible formation of D-arabinose isomerase, but competitively inhibit intracellular D-arabinose isomerase reaction. In consequence, growth is inhibited specifically.

Recently, an interesting result was reported by Reiner (11), i.e., that D-arabitol was toxic to many laboratory strains of *Escherichia coli* K-12, and xylitol was found to be toxic to an

existing *E. coli* C mutant strain. The mechanism of toxicities was postulated due to derepressed fructose, galactitol, and sorbitol phosphotransferases. Phosphorylated xylitol and D-arabitol seemed to be toxic compounds. The mechanism reported in this paper is different from that described by Reiner in *E. coli*, but is not inconsistent. How a pentitol-acting phosphotransferase system is induced in *K. aerogenes* M-7 is an interesting problem and one which we are in the process of studying.

Several kinds of mutant strains that are resistant to pentitols may be isolated. For example, mutants (i) that can metabolite pentitols, (ii) that can metabolite the pentose by a new metabolic pathway, (iii) that produce a new pentose isomerase which is insensitive for pentitols, and (iv) that produce pentose isomerases in a great amount, should be resistant to pentitols. We will be able to utilize these phenomena in selecting a mutant that produces a certain pentose isomerase in a great amount.

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