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A Unique Pattern of Toxic Synthesis in Pentitol Catabolism: Implications for Evolution

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Summary. All of our *Escherichia coli* C mutants blocked in the first step of D-arabitol catabolism (D-arabitol dehydrogenase) became unable to grow in the presense of D-arabitol. We have shown that this sensitivity is eliminated by a defect in the second enzyme of the pathway (D-xylulokinase), leading to a pattern of toxicity and its relief which has not been previously reported. We have found a similar pattern of toxicity and its relief in the closely related ribitol pathway. The evolutionary significance of these findings is discussed.

Key words: D-arabitol-ribitol-toxic synthesis-pathway evolution

Introduction

Toxic or lethal synthesis is the enzymatic conversion of a harmless substance into a toxic one. In bacteria, such synthesis normally occurs in catabolic pathways in which a blockage subsequent to a phosphorylation step leads to the accumulation of a toxic, phosphorylated intermediate. Many such examples of toxic synthesis have been reported, including certain mutants in the galactose, fructose, mannitol, and L-arabinose pathways (Englesburg et al., 1962; Ferenci and Kornberg, 1973; Lengeler, 1975; Solomon and Lin, 1972; Yarmolinski et al., 1959). In humans, classic cases of inborn errors of metabolism such as galactosemia (Segal, 1972) and hereditary fructose intolerance (Froesch, 1972) are the result of analogous lethal syntheses of sugar phosphates. Instances of lethal syntheses which have been characterized in wild type organisms include chlorate to chlorite conversion in bacteria (Piechaud et al., 1967), acrolein synthesis by yeast (Wills and Phelps, 1975) and mannose to mannose-phosphate conversion by honeybees (Sols et al., 1960).

Since the toxic compounds in carbohydrate catabolic pathways are usually phosphorylated ones, a block prior to a phosphorylation normally does not result in lethal sythesis. In our studies of pentitol catabolism in *E. coli* C, however, we found that all of 35 indepedent mutants blocked in the *first* enzyme of D-arabitol catabolism (Fig. 1) are prevented from growth if D-arabitol is present. Our results show that this inhibition is overcome by a mutation in the *second* enzyme of the pathway, leading to a

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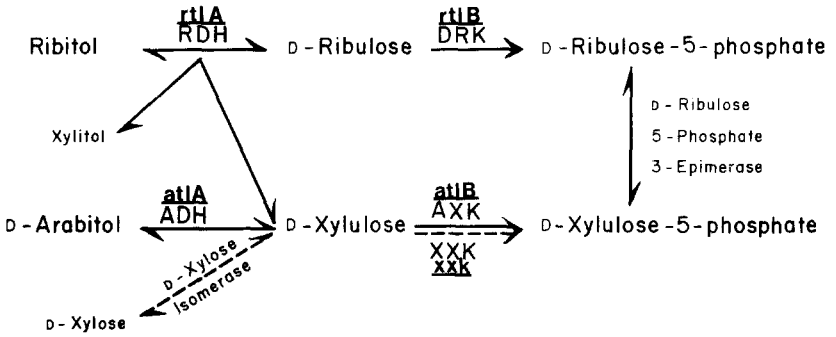

Fig. 1. The pentitol catabolic pathways of *E. coli*. The pathways are found in less than 10% of natural *E. coli* isolates and in the laboratory strain C, although not in strains B or K-12 (Reiner, 1975). They can be transferred by transduction into K-12 strains. In transductions between *E. coli* C and K-12, the ribitol and D-arabitol genes are 100% linked, as expected if *E. coli* K-12 strains lack DNA homologous to the pentitol loci. This allows the transduction of negative mutants of one pathway by selection for the other. Wild type *E. coli* metabolizes xylose via xylulose, and produces a xylulokinase (XXK) distinct from AXK. When AXK activity is blocked, XXK is induced in response to D-arabitol and so can substitute for AXK

pattern of toxicity and its relief which has not been previously reported. That activity of the second enzyme of a pathway on the substrate of the first can lead to toxicity may indicate that its avoidance may be a significant evolutionary force. Such activity also is consistent with the gene duplication hypothesis of Horowitz (1945).

Materials and Methods

Bacterial Strains. Sources and properties of bacterial strains are described in Table 1.

Growth of Bacteria. Minimal, YT and YTG media are as described previously (Scangos and Reiner, 1978). CH medium is 5 g/l casein hydrolysate (vitamin free, Difco) supplemented with 15 g/l agar for plates.

Determination of Sensitivity. Sensitivity to ribitol and D-arabitol was tested by streaking a strain on CH and on CH supplemented with one of the compounds at the indicated concentration. Sensitive strains grow poorly, or not at all on supplemented plates while resistant strains grow at least as well on the supplemented plates as on the CH plates.

Isolation of Resistant Mutants. Sensitive strains were grown from single colonies in YT broth to a density of 10^9/ml and 0.1 ml was plated on CH supplemented with the desired compound. Resistant mutants, which appeared as colonies capable of growth in the presence of the pentitol, were purified by streaking and retested.

Isolation of Temperature Sensitive Strains. Temperature sensitive mutants of ADH were obtained by two methods. The first was to isolate at 30° D-arabitol$^+$ revertants of ADH$^-$ strains and screen for the inability to grow at 40°. The second method was a penicillin selection performed directly at 40°. ADH$^-$ mutants from such a selection were scored for their ability to grow at 30° on D-arabitol.

Table 1. Bacterial Strains

Strain	Genotype	Source
XK100	*pro* XXK$^-$	Nitrosoguanidine (NTG) mutagenesis of PK191 (from B. Bachmann)
XK103	*pro* XXK$^-$ *rt1A*	P1 transduction of XK100
XK113	*pro* XXK$^-$ *at1A rt1A*	NTG mutagenesis of XK103
XK113A	*pro* XXK$^-$ *at1A rt1A At1*^{R}	spontaneous derivative of XK113
XK133	*pro* XXK$^-$ *at1A rt1A*	NTG mutagenesis of XK103
GM301	*met rt1A* Rt1 constitutive	derivative of CGSC 4312
C50	Ura$^-$ *at1A* (ts)	NTG mutagenesis of C131

List of abbreviations: XXK$^-$ – defect in the gene for D-xylose induced xylukokinase; rt1A – gene for ribitol dehydrogenase (RDH); at1A – gene for D-arabitol dehydrogenase (ADH); At1^R – resistance to D-arabitol; Ura$^-$ – requirement for uracil

Miscellaneous Techniques. Methods for the isolation of ribitol and D-arabitol negative mutants, in vitro assays of enzymatic activity, conjugation, phage Pl-mediated transduction and the selection and scoring of constitutive strains have been previously described (Scangos and Reiner, 1978).

Results

Genetic Evidence that the Mutation to D-arabitol-resistance Lies Within the D-arabitol Pathway. All 35 of our *Escherichia coli* mutants blocked in D-arabitol dehydrogenase (ADH), the first enzyme in D-arabitol catabolism (Fig.1) become both D-arabitol negative (are unable to utilize D-arabitol as a carbon and energy source) and sensitive (are unable to grow on other substrates if D-arabitol is present). Spontaneous mutants of these ADH$^-$ strains which remain D-arabitol negative but become insensitive are readily obtainable. We showed by comparing reversion rates that these mutations which relieve D-arabitol sensitivity also occur within the D-arabitol catabolic pathway. ADH$^-$ strains generally revert to D-arabitol$^+$ (Atl$^+$) (i.e., regain the ability to grow on D-arabitol) at frequencies between 10^{-6} and 10^{-9}. D-arabitol-resistant derivatives of ADH$^-$ strains do not revert to Atl$^+$ at detectable frequency ($<2X10^{-10}$), as expected of double mutants in the D-arabitol pathway. These D-arabitol-resistant mutations do not affect growth on other carbohydrates.

Mutations within the D-arabitol pathway are genetically closely linked to each other and to the ribitol loci (Scangos and Reiner, 1978). We confirmed that the mutation relieving the lethal synthesis was extremely closely linked to the mutation causing it: all 50 strains to which we transferred the one mutation by phage Pl-mediated generalized transduction also received the other mutation. The Rtl$^-$ Atl$^+$ strain XK103 was transduced to Rtl$^+$ from the Rtl$^+$ ADH$^-$ Atl-resistant donor XK113A. Of 50 Rtl$^+$ recipients which received the ADH$^-$locus, all received the Atl-resistant locus as well.

Enzymatic Evidence that AXK is Lost in D-arabitol-resistant Mutants. The only known step specific to D-arabitol catabolism, other than ADH, is the D-arabitol-induced

Table 2. Enzyme activities of D-arabitol dehydrogenase mutants and their arabitol-resistant derivatives[a]

Strain	Relevant properties	ADH[b]	AXK[b]
XK113	ADH^- Atl^s	< 0.005	0.24
XK113A	ADH^- $at1^r$	< 0.005	<0.01
XK133	ADH^- $at1^s$	<0.005	0.25
XK133A	ADH^- $at1^r$	<0.005	<0.01
C50	ADH^{ts} $at1^s$ at 43^o	0.025	0.45
C50 A-1	ADH^{ts} $At1^r$	0.018	0.01
C50 A-2	ADH^{ts} $At1^r$	0.021	0.01

[a] Cells were grown in CH supplemented with 2 mM D-arabitol;
[b] Units are expressed as μmoles NADH oxidized per min/mg protein. Measured ADH activity in strain C50 and its derivatives is much below wild type, presumably because of instability of the temperature-sensitive enzyme

xylulokinase (AXK, Fig. 1). (No mutants defective in D-arabitol permeation have been reported among over 60 D-arabitol negative mutants isolated by us and others. It is therefore doubtful whether a single mutation can abolish D-arabitol permeation). A comparison of AXK activity in two D-arabitol-sensitive strains, XK113 and XK133, and their D-arabitol-resistant derivatives, is shown in Table 2. It can be seen that the D-arabitol-resistant derivatives have lost all demonstrable AXK activity. This loss of AXK activity could result either from a mutation in the AXK structural gene, or from a control or permease mutation which shuts down the entire pathway. Since the D-arabitol-sensitive strains are necessarily blocked in ADH, some care must be taken to distinguish these possibilities.

Proof that a Mutation in the Structural Gene for AXK Leads to D-arabitol Resistance. To distinguish structural gene mutations from control or permease mutations we used mutant C50, whose ADH is temperature sensitive: at 43^o the strain is ADH^- and D-arabitol-sensitive, and at 30^o it is ADH^+ and D-arabitol-insensitive. Derivatives of this strain which are selected for D-arabitol-resistance at 43^o would retain ADH function at 30^o if the resistance were due to a mutation in the structural gene for AXK, but would lose ADH function at 30^o if resistance were due to a mutation which shuts down the entire pathway.

For this purpose, strain C50 was plated at 43^o on CH plates supplemented with 8 mM D-arabitol. We were conveniently able to score those resistant mutants which retained ADH activity because they could grow on D-arabitol at 30^o in the absence of AXK by utilizing thexylulokinase from the xylose pathway (XXK, Fig. 1). Approximately half of the small, D-arabitol-resistant, mutant colonies which arose remained capable of growth on D-arabitol at 30^o, as expected only of mutants in the AXK structural gene. (The inability of these strains to grow on D-arabitol at 43^o distinguished them from revertants of the original ADH defect).

That these D-arabitol-resistant mutants were indeed AXK structural gene mutants was verified both genetically and biochemically. Growth on D-arabitol in the absence of AXK is specifically dependent on XXK activity. Therefore, when transferred into a XXK^- strain, the D-arabitol pathways of the resistant derivatives should no longer confer growth on D-arabitol at 30^o. (The D-arabitol phenotype should not be altered by the absence of XXK if a control or permease mutation has led to D-arabitol-resistance). The D-arabitol pathways of two independent C50 D-arabitol-resistant derivatives (Atl^+ at 30^o) were transferred by transduction into the *E. coli* K-12 XXK^- strain XK100 by selection on ribitol (Reiner, 1975). As predicted, in the absence of XXK, growth on D-arabitol no longer could occur. (The D-arabitol pathway of the D-arabitol-sensitive, AXK^+ parent does confer growth on D-arabitol at 30^o after transduction into the same strain). Enzyme levels of ADH and AXK in the temperature sensitive ADH mutant and its D-arabitol-resistant derivatives also confirm the specific loss of AXK in the resistant mutants (Table 2). Both D-arabitol-resistant strains lost the AXK activity of their parent strain, while retaining ADH activity. (Control or permease defects would be expected to affect both ADH and AXK activities equally). Thus, the elimination of AXK relieves the D-arabitol toxicity caused by a block in the prior enzyme, ADH.

A Similar Pattern is Found in the Ribitol Pathway. We suspected that this same novel pattern of lethal synthesis and its relief might occur in the ribitol catabolic pathway. This pathway (Fig. 1) closely resembles that of D-arabitol, except that the intermediate (ribulose) rather than the pentitol itself is the inducer (Scangos and Reiner, 1978). Therefore, in this case a toxic synthesis caused by the kinase would be excepted only in constitutive strains. We isolated an RDH^- mutant strain from the ribitol constitutive strain GM301 and indeed found it to be sensitive to ribitol. (On the other hand, none of the 16 RDH^- mutants isolated from inducible parents were ribitol sensitive). Three independent ribitol-sesistant derivatives of the RDH^- constitutive strain, selected in the presence of 25 mM ribitol, lost their DRK activity (Table 3). It thus appears that in the ribitol pathway, as in the D-arabitol pathway, the presence of the kinase in the absence of the dehydrogenase causes sensitivity to the pentitol. Induction by an intermediate, so that sensitivity is found only among constitutive strains, is clearly the more advantageous for avoiding toxicity.

Table 3. Enzyme activites of a ribitol dehydrogenase ribitol constitutive mutant and its ribitol-resistant derivatives[a]

Strain	Relevant properties	RDH[b]	DRK[b]
GM301	$RDH^- Rtl^{Cc}$ Rtl^{Sd}	<0.001	0.6
$GM301R_1$[e]	$RDH^- DRK^-$ Rtl^R	<0.001	<0.05
$GM301R_2$	$RDH^- DRK^-$ Rtl^R	<0.001	<0.05
$GM301R_3$	$RDH^- DRK^-$ Rtl^R	<0.001	<0.05

[a] Cells were grown in CH supplemented with 2 mM-arabitol; [b] Units are expressed as μmoles NADH oxidized per min/mg protein; [c] Constitutively synthesizes DRK; [d] Sensitive to ribitol
[e] GM301-R_1, R_2, and R_3 are independent ribitol resistant derivatives of GM301

Discussion

The classic cause of toxic or lethal synthesis is a mutation in an intermediate metabolic step, subsequent to a phosphorylation reaction, which leads to the accumulation of a phosphorylated compound. The classic relief of lethal synthesis is a mutation in a prior step of the pathway which prevents the accumulation of the toxic, phosphorylated compound.

Toxic synthesis in the D-arabitol and ribitol pathways of *E. coli* differs from the usual pattern in two ways. A block in the first enzyme, *prior* to a phosphorylation reaction, leads to the accumulation of a toxic product. The toxicity is then relieved by a mutation in a *subsequent* step rather than a prior step. Because this subsequent step itself is a phosphorylation reaction, and because both D-arabitol phosphate and ribitol phosphate elsewhere have been implicated in toxic synthesis in *E. coli* (Katz, 1970; Reiner, 1977), the kinases of the D-arabitol and ribitol pathways almost certainly are responsible for the lethal syntheses observed here. We believe that with dehydrogenase activity blocked, D-arabitol or ribitol at abnormally high intracellular concentrations serve as substrates for the kinases, and so are phosphorylated at low levels to the corresponding toxic pentitol phosphate. When the kinase activity is lost, toxicity is eliminated. The previously noted ability of pentulose kinases to act on more than one substrate (Leblanc and Mortlock, 1972; Mortlock, 1976) further supports this hypothesis.

Activity of an enzyme on other substrates in the same pathway clearly can be disadvantageous, and in our example can be sufficient to abolish growth of the organism altogether. Although we see this activity most strongly in negative mutants, the activity may affect the growth of wild type cells as well.

Toxic syntheses due to such non-specific reactions may represent a strong and general evolutionary force. Sequential substrates within a pathway are most often closely related compounds. Enzymes which catalyze sequential reactions might therefore be expected to possess, at least initially, activity on prior or subsequent substrates. Since such non-specificity seriously affects growth of cells under certain conditions, its avoidance may represent a significant evolutionary force. Enzymes may have to reach a balance between maximum activity on their intended substrates and minimum activity on related ones.

Yeh et al. (1978) recently have shown that sequential steps within the β-ketoadipate pathways of *Acinetobacter* and *Pseudomonas* most likely have arisen by gene duplication. Interestingly the two enzymes (Muconolactone isomerase and enol-lactone hydrolase II) within *Acinetobacter* have diverged widely while *Acinetobacter* muconolactone isomerase and *Pseudomonas* enol-lactone hydrolase II retain significant homology. This is the expected result if divergence of sequential enzymes within a pathway is favored.

Our data are also consistent with the classic evolution hypothesis proposed by Horowitz (1945) and modified by Lewis et al. (1951) whereby the sequential steps within a pathway evolve one from the other by gene duplication followed by mutation. In this scheme, the newly evolved enzyme is expected to retain low level affinity for its original substrate, such as the kinases apparently do for the pentitols. This hypothesis is probably true for a minority of pathways (Hegeman and Rosenberg, 1970; Jeffcoat and Dagley, 1973) and need not be invoked to explain the enzyme activities we see here. Nevertheless, it is consistent with our data and interesting in view of the experimentally induced duplications of the ribitol operon by two laboratories (Rigby et al., 1974; Inderlied and Mortlock, 1977).

It has been speculated that the ribitol and D-arabitol genes, which are closely linked and lie in a mirror image arrangement, evolved one from another by gene duplication and inversion (Inderlied and Mortlock, 1977). Thus, the four enzymes of pentitol catabolism conceivably may have arisen from a single common ancestral gene by sequential duplications. Experiments to test this hypothesis directly by determining the extent of DNA homology among the genes of pentitol catabolism are currently in progress. Preliminary data indicate that there may be regions of homology between the two operons.

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