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Accumulation of Toxic Concentrations of Methylglyoxal by Wild-Type *Escherichia coli* K-12

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Wild-type strains of *Escherichia coli* K-12 accumulate toxic concentrations of methylglyoxal when grown in medium containing adenosine 3',5'-monophosphate and either D-xylose, L-arabinose, or D-glucose-6-phosphate, independent of the presence of other carbon sources. Mutations at a locus called *cxm* specifically block methylglyoxal formation from xylose in the presence of adenosine 3',5'-monophosphate. Accumulation in medium containing xylose, studied in some detail, is dependent on the ability to utilize xylose and is associated with an increased rate of xylose utilization without changes in levels of xylose isomerase. These results suggest that adenosine 3',5'-monophosphate results in induction of excessively high levels of an early rate-limiting step in xylose metabolism. This step may be the transport of xylose into the cell. The resulting excessive rates of xylose catabolism could stimulate methylglyoxal formation by overburdening late steps in glycolysis.

The central position of the Embden-Meyerhof pathway in the metabolism of sugars by enterobacteria is well established. Other pathways, however, do exist. In one of these (Fig. 1), methylglyoxal (MG) is an intermediate in a series of reactions leading from dihydroxyacetone phosphate (DHAP) to pyruvate. This MG bypass of the terminal steps of glycolysis is found in many enterobacteria (8). The MG pathway must be carefully regulated because MG concentrations of 0.5 mM and higher are toxic to cells (4).

Production of toxic amounts of MG by bacteria has been reported in only a single case, that of a mutant strain of *Escherichia coli* in which the addition of glycerol led to the accumulation of MG (6, 12). We have found, in the course of studies on the reversal of catabolite repression by adenosine 3',5'-monophosphate (cAMP), that accumulation of MG occurs in wild-type *E. coli* K-12 when the cells are grown in medium containing cAMP and one of a number of carbon sources. In this report we describe the kinetics of growth inhibition, show that the toxic compound which accumulates is MG, and suggest a possible mechanism for MG accumulation. (Part of this work is from a dissertation submitted by R. S. A. to the University of Chicago in partial fulfillment of the requirements for the degree of Master of Science, June 1972.)

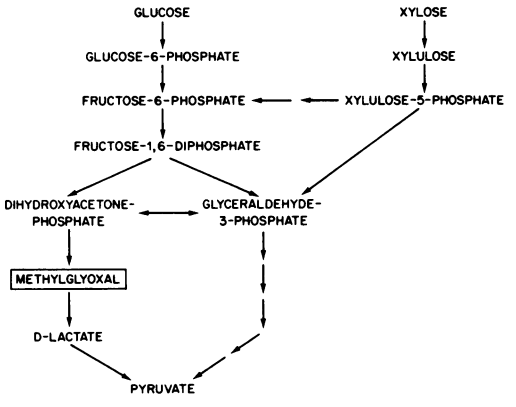

FIG. 1. *An outline of glycolysis showing the methylglyoxal bypass.*

MATERIALS AND METHODS

Media. Cells were grown in a phosphate-buffered minimal medium referred to as K115 (5). Carbon sources were present at 2 g/liter and thiamine was present at 1 mg/liter. Mutant strain X9003R was isolated on agar plates of K115 medium containing 5 g/liter each of D-xylose and glycerol, and 2 mM cAMP.

Bacterial strains. *E. coli* K-12 strain X9003 (F^- *thi* $lacZ_{M15}$) was used as the wild-type strain. Strain X9003R, a spontaneous mutant of X9003 which does not produce MG in medium containing xylose and cAMP, was isolated on the plates described above.

MG assays. The assay with glyoxalase was performed at room temperature as described by Racker (16).

The 2,4-dinitrophenylhydrazine (2,4-DNPH) derivatives were prepared by incubating 1 ml of sample with 0.33 ml of 0.1% (wt/vol) 2,4-DNPH in 2 N HCl at 30 C for 15 min. The spectra of the products were measured after the addition of 1.67 ml of 2.5 N NaOH and a 15-min incubation at room temperature (1). For chromatography, the reaction mixtures were extracted with ethyl acetate. After concentration by evaporation, the extracts were separated on Whatman #1 paper in the descending direction using *n*-butanol equilibrated with 1.5 M NH_4OH. After drying, the chromatograms were sprayed with a solution of 10 g of NaOH in 100 ml of 60% ethanol.

A bioassay for MG utilized the growth inhibition produced by this compound. An exponential-phase culture of strain X9003R in minimal-xylose-glycerol medium was centrifuged, washed, concentrated, and added to a series of tubes containing fresh xylose-glycerol minimal medium and varying amounts of either MG or a concentrated supernatant from a culture that had reached growth arrest in xylose medium with cAMP. In those tubes receiving large amounts of the culture supernatants, the minimal medium was diluted with water to maintain approximately the same phosphate concentration in all tubes. The turbidity was measured initially, and after 150 min of incubation with shaking at 37 C. A standard curve relating the logarithm of the increase in turbidity to the amount of MG added was linear over a range of MG concentrations from 0.14 to 1 mM. From this curve, the equivalent concentration of MG in the culture supernatants was determined. Strain X9003R was used in the assay to prevent production of MG by the assay strain during the 150 min of the test.

Xylose and enzyme assays. Xylose consumption was measured by collecting culture filtrates, absorbing with activated charcoal to remove cAMP, treating with 0.1 N NaOH for 30 min at 30 C to destroy MG, neutralizing with HCl, and then assaying for pentoses by the carbazole method (7). Slight interference by the alkaline degradation product of MG was eliminated by reading samples and standards at 530 and 400 nm, the absorption maximal of the xylose and MG degradation product derivatives, respectively. From the relative absorption at these two wave lengths for the standards, xylose concentrations in the samples were calculated.

The initial rate of xylose uptake was measured in cells, collected by filtration, washed, suspended at approximately 200 μg (dry weight) per ml in substrate-free K115 containing 50 μg of chloramphenicol per ml, and aerated for approximately 10 min at 30 C. Uptake measurements were initiated by adding 10^{-4} M D-[1-^{14}C]xylose. Samples were collected approximately 12 and 24 s later on 25-mm membrane filters (0.45 μm pore size, Millipore type HA) overlayed with 2 ml of ice-cold 0.4 M glucose. Suction was applied as the samples were added, and the filters were washed twice with 2 ml of cold 0.4 M glucose, dried, and counted in a liquid scintillation spectrometer. Uptake rates were calculated from these two early time points. Control experiments showed that uptake was linear under these conditions for at least 90 s. The turbidity of the cell suspensions was measured at 610 nm and converted to dry weight values by use of a previously established calibration curve.

Xylose isomerase was assayed in sonic extracts clarified by centrifugation at 35,000 $\times$ *g* for 30 min. The clarified extracts were incubated at 37 C with 20 mM D-xylose in 0.1 M sodium borate buffer (pH 8), and the rate of formation of ketose was measured by the cysteine carbazole method (2, 3). One unit of isomerase results in an increase in absorbancy at 540 nm of 1 per min in the above assay system where the final volume is 6 ml. Protein was measured by the Folin phenol method (13), using bovine serum albumin as standard.

Chemicals. D-[1-^{14}C]xylose was obtained from Calatomic; D-xylose was from Difco; cAMP, sodium pyruvate, MG, GSH, and glyoxalase I (630 units/mg of protein) were from Sigma Chemical Co., St. Louis, Mo. MG was purified by passage through a Dowex-1 chloride column.

RESULTS

This investigation began with the chance observation that *E. coli* K-12 does not grow in minimal medium containing D-xylose as carbon source if cAMP is present in concentrations of approximately 0.5 mM or higher. In liquid culture, the addition of cAMP arrests growth within 2.5 h (Fig. 2A). After growth arrest, turbidity is constant for many hours, but cell viability falls rapidly (Fig. 2B). This phenomenon is associated with the appearance of a toxic substance in the culture supernatant, since such supernatants cause immediate growth inhibition and killing of exponentially growing cells.

Growth arrest by D-xylose and cAMP occurs even if other carbon sources are added. When glycerol is also present, the changes in culture turbidity and viable count are virtually superimposable with those of Fig. 2. Somewhat higher concentrations of cAMP are needed to produce growth arrest when glucose or peptone are present. Growth arrest is dependent on the ability of the cell to metabolize xylose; several *xyl* mutants grew in glycerol-xylose medium containing cAMP.

Mutants resistant to growth inhibition by xylose and cAMP arise spontaneously at high frequency. Such mutants are readily isolated by streaking cultures on the xylose-glycerol-cAMP plates described in Materials and Methods. We had expected to isolate *xyl* mutants in this way, but found instead that the vast majority of the mutants are *xyl*$^+$ and carry a mutation at a locus we call *cxm*, for cAMP xylose methy-

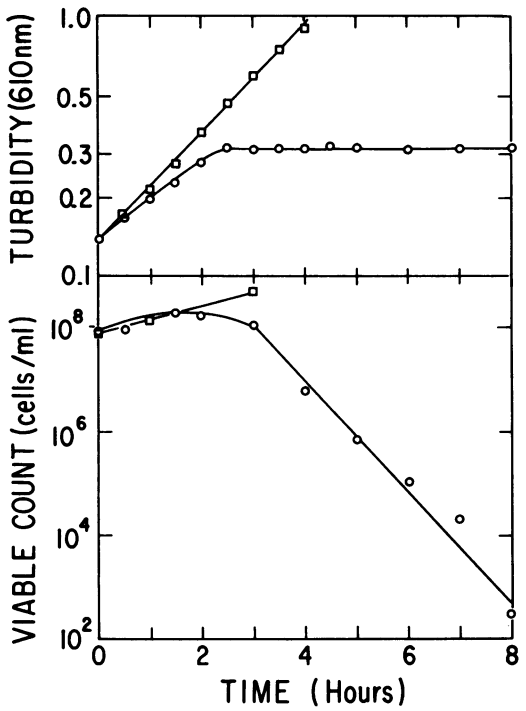

FIG. 2. *Kinetics of growth inhibition and killing by xylose and cAMP. A culture of strain X9003 growing in 0.2% xylose K115 medium at 37 C was divided at time zero, one part* (○) *receiving 2 mM cAMP while the other* (□) *served as control. (A) The culture turbidity measured in 18-mm test tubes at 610 nm in a Bausch and Lomb Spectronic colorimeter. (B) The number of viable cells per milliliter.*

glyoxal production. The *cxm* mutants do not produce the toxic substance, but are just as sensitive to its action as are wild-type cells. Selection for *cxm* mutants must be performed on solid medium. In liquid medium the toxic product has access to all cells regardless of which cells produce it, whereas on plates its movement is limited by diffusion. Another factor is that MG diffusing from nearby cells will be inactivated by the glyoxalase of the cells in the outer layers of a colony, protecting cells near the center.

Characterization of the toxic product as MG. The occurrence of growth arrest and killing without cell lysis, due to a toxic substance produced by the cells, was reminiscent of the effects of MG produced from glycerol in a mutant strain of *E. coli* (6, 12). Our toxic substance has the same stability properties as reported for MG: it is stable to acid (pH 3.5, 37 C, 30 min) but is destroyed by boiling at neutral pH for 30 min, or by treatment with alkali (pH 11.5, 37 C, 30 min). The spectrum in alkali of the 2,4-dinitrophenylhydrazine derivative from a toxic supernatant is very similar to that obtained with authentic MG, both having a peak at 550 nm.

The 2,4-DNPH derivatives were analyzed by chromatography to characterize these compounds. The wild-type supernatant showed three spots: an orange spot, R_f 0.55; a yellow spot, R_f 0.64; and a brown spot, R_f 0.74. The derivative of MG showed only a single orange spot with an R_f of 0.56, while pyruvate gave two spots, a yellow spot with an R_f of 0.65, and a brown spot with an R_f of 0.74. Supernatants from a *cxm* mutant showed only the two spots found for pyruvate.

Enzymatic confirmation of the accumulation of MG was obtained by assaying for this compound with glyoxalase I, an enzyme specific for ketoaldehydes (16). From the supernatants exhibiting toxicity, rather large concentrations of MG were measured by this assay: 1.42 mM in the case of one supernatant, and 4.8 and 9.3 mM for two supernatants, concentrated 5 to 10-fold by evaporation. The concentrated supernatant from the *cxm* mutant contained no detectable MG.

All of the toxic activity of our inhibitory supernatants can be accounted for by their MG content. The supernatant that contained 4.8 mM MG by assay with glyoxalase was equivalent in growth inhibition by bioassay to 4.3 mM MG. Further, treatment of toxic supernatants with glyoxalase and glutathione completely abolished their inhibitory effects.

Production of MG was also observed in cAMP containing media when either L-arabinose or D-glucose-6-P were present. Kinetics of inhibition with glucose-6-P were similar to those with xylose, while with arabinose, higher concentrations of cAMP were needed and growth arrest began somewhat later. Production of MG may also occur with several other carbon sources, since growth yields on L-rhamnose, D-fructose, and mannitol are lower in medium containing cAMP than in medium containing only the sugar.

The *cxm* mutation prevents MG production from xylose but does not alter MG production from arabinose or glucose-6-P. The biochemical function of the *cxm* gene product is not known. The *cxm* locus, originally referred to as *cxr* and so listed on the latest map of *E. coli* (19), is between the *proA* and *lac* loci near min 7 on the map. Deletions which remove *cxm* result in the Cxm phenotype, indicating that this property is due to absence of a functional *cxm* gene product

(R. Ackerman, J. Golin, and W. Epstein, manuscript in preparation).

Mechanism of MG accumulation. The role of xylose metabolism in MG accumulation was investigated by measuring uptake and utilization of xylose and levels of xylose isomerase in wild-type and *cxm* mutant strains. A plot of xylose concentration remaining in the medium versus time showed no consistent differences between strains or any effect of cAMP (Fig. 3A), but when the same data were plotted versus bacterial mass as estimated from turbidity, an increased rate of utilization is seen in strain X9003, beginning approximately 30 min after the addition of cAMP. The effect is not large and, from the data shown, might be considered due to scatter of the data, but two other experiments of this type showed increases of 36% and 40% in the rate of xylose consumption in strain X9003 after cAMP addition. As expected from the results of Fig. 3, cAMP stimulates the rate of xylose uptake in strain X9003, but does not have a significant effect in strain X9003R (Table 1). No significant effects on levels of xylose isomerase were seen in either strain (Table 1).

DISCUSSION

Cooper and co-workers (1, 8, 9) recently demonstrated that *E. coli* and several other bacteria have a non-phosphorylating pathway from triose phosphate to pyruvate and that MG is an intermediate in this pathway (Fig. 1).

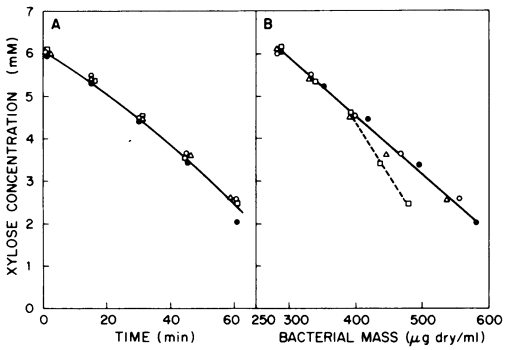

FIG. 3. *Effect of cAMP on xylose utilization. Exponential-phase cultures of strains X9003 and X9003R growing at 37 C were divided at zero time, one half serving as control while the other received 4 mM cAMP. Culture filtrates were obtained at 15-min intervals and assayed for xylose as described under methods. (A) The data are plotted versus time; (B) the same data are plotted versus bacterial mass. Symbols: ○, X9003 control; □, X9003 with cAMP; △, X9003R control; ●, X9003R with cAMP.*

TABLE 1. *Effect of cAMP on xylose uptake and xylose isomerase*

Strain	cAMP		Xylose uptake[a] (μmol/g/min)	Xylose isomerase[b] (units/mg protein)
	Concn (mM)	Min after addition		
X9003	0		43.8 ± 2.5	5.45
			43.3 ± 1.7	5.95
	4	56	52.3 ± 2.6	
	4	66	53.2 ± 2.0	
	2	78		5.74
X9003R	0		50.2 ± 1.4	5.25
	2	64	46.1 ± 4.3	5.15

[a] Each value is the average and standard error of the mean for four replicate determinations.

[b] Isomerase assays were performed using different cultures than those used for uptake measurements. The last value is from a culture of X9003R growing for over 10 generations in xylose cAMP medium.

These workers have described some unusual regulatory properties of MG synthase, the key enzyme which forms MG from DHAP. Based on the properties of MG synthase we can suggest a plausible explanation for MG accumulation from xylose in the presence of cAMP. Both MG synthase which forms MG and glyoxalase which degrades it are present constitutively in *E. coli*, but the level of the former activity is approximately an order of magnitude higher than that of the latter (6, 8). Therefore MG synthase must be sharply inhibited if MG accumulation is to be avoided. MG synthase activity is probably kept in check by inorganic phosphate which markedly inhibits the enzyme. Phosphate inhibition is cooperatively overcome by increasing the substrate concentration (8, 9), so that a sufficient elevation of DHAP pools should lead to rates of MG synthesis exceeding the rate at which glyoxalase can degrade MG. One example of this situation may be the rapid production of toxic levels of MG in a mutant of *E. coli* (6, 12). The mutant has high constitutive levels of the glycerol catabolic enzymes, and its glycerol kinase is insensitive to feedback inhibition by fructose 1,6-diphosphate. Missing two of the major restraints on glycerol catabolism, the mutant rapidly converts glycerol to DHAP, and if that rate is higher than the rate at which DHAP can be further metabolized, the cell pool of DHAP will rise and stimulate MG synthesis.

Increased xylose utilization after cAMP addition suggests that this nucleotide acts to stimulate a rate-limiting step early in xylose metabo-

lism. The *cxm* mutation prevents formation of MG from xylose and appears to do so by preventing the increase in xylose uptake and consumption produced by cAMP in wild-type strains (Fig. 3, Table 1). The fact that this mutation does not prevent MG production from arabinose argues that the cAMP-affected step is specific to xylose. Only the first two enzymatic steps in xylose metabolism, leading to formation of xylulose-5-P, are specific to xylose; later degradative steps of pentose metabolism are common to xylose and arabinose. Since no change is seen in xylose isomerase, and the isomerase and xylulokinase appear to be coordinately regulated in *E. coli* (2), it is unlikely that the effects are mediated by changes in the levels of either of these soluble xylose-specific enzymes. It is therefore probable that the primary effect of cAMP is to increase xylose transport into the cell, a conclusion consistent with the measured increase in initial rate of xylose uptake.

Since the only documented effect of cAMP in bacteria is the regulation of transcription from certain genes, especially those for inducible enzymes of sugar catabolism (15), such can be assumed to be the role cAMP plays here. Submaximal induction of a xylose transport system, postulated to be the specific step reversed by cAMP, is probably an example of self-catabolite repression, a term describing repression mediated by cAMP and produced by a carbon source on the enzymes and transport system needed for its metabolism (11, 14). Stimulation of xylose uptake could shift the rate-limiting step for xylose metabolism to a point after the level of triose phosphate. Then DHAP would accumulate and stimulate MG formation. Stimulation of transport and a shift in control from an early to a relatively late step can also explain MG formation from glucose-6-P. The only step specific to glucose-6-P metabolism is a transport system whose synthesis is regulated by cAMP (J. A. Ezzell and W. J. Dobrogosz, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1972, p. 176).

This model for MG formation indicates that, for those carbon sources which can lead to MG formation, an important control mechanism is self-catabolite repression of transport into the cell. For sugars which do not lead to MG accumulation in the presence of cAMP, other control mechanisms such as feedback inhibition, or regulation of the degree of induction through effects on inducer metabolism, must work to maintain an early step as rate-limiting. It may be predicted that removal of such a control by mutational loss of feedback inhibition would allow MG production in the presence of cAMP from carbon sources where such accumulation does not occur in wild-type bacteria.

There is an interesting correlation between the presence of MG synthase and the presence of cAMP in different bacterial species. Both are present in all enterobacteria examined and both are absent in *Bacillus subtilis* (8, 10, 18). If such a correlation is confirmed by studies of other bacteria, it would suggest that in bacteria which have MG synthase, cAMP confers a selective advantage by preventing formation of toxic levels of MG from some carbon sources.

Selection for mutants which do not produce MG in the presence of cAMP is a potentially useful method. As noted above, selection in medium containing xylose was disappointing since the vast majority of mutants obtained were the *cxm* class. Both spontaneous and mutagen-induced mutants defective in cAMP binding protein have been obtained when cells of strain LU53 (17) are plated on minimal glucose-6-P medium containing cAMP. Some of these may be *crp* deletions since they have not been observed to revert. Selection on plates containing cAMP and all three of the MG-producing carbon sources yields a number of different mutant types, most of which have not yet been characterized. One common type is a *crp* mutation leading to an altered but partially functional cAMP binding protein. No mutants with changes in MG synthase have been identified to date (F. Sudborough, unpublished observations).

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