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Ammonia-Sensitive Mutant of *Klebsiella aerogenes*

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We have isolated a temperature-sensitive mutant of *Klebsiella aerogenes* unable to grow aerobically at 42 C in standard glucose minimal medium containing 0.03 M ammonium sulfate as a source of nitrogen. This strain, MK810, will grow at this temperature in significantly lower concentrations of ammonia (1 mM) or when ammonia is replaced by a growth rate-limiting source of nitrogen such as histidine or glutamate. A detailed physiological characterization and preliminary biochemical tests support the contention that the mutant has an altered α-ketoglutarate dehydrogenase that at the restrictive condition fails to manufacture sufficient succinyl-coenzyme A. We explain the ammonia sensitivity by the dual role of α-ketoglutarate as substrate for the formation of succinyl-coenzyme A and glutamate. A defect in the enzyme necessary for the production of succinyl-coenzyme A makes ammonia an overly effective competitor for α-ketoglutarate.

In the course of our studies of glutamine synthetase in *Klebsiella aerogenes* (6), we considered the possibility that a mutant incapable of converting glutamine synthetase to its inactive adenylylated form (8) might fail to grow in media containing ammonia at the usual concentration of 0.03 M but would grow in media containing ammonia in much lower concentration or some other nitrogen source. The reason for this sensitivity to ammonia would be the adenosine 5'-triphosphate deficiency resulting from excessive glutamine synthesis (11). We therefore undertook the isolation of an ammonia-sensitive mutant, a phenotype that so far has not been described. The characteristics of the temperature-sensitive mutant we obtained are described in this paper. The mutant is not defective in glutamine synthetase, but rather in the α-ketoglutarate dehydrogenase complex. The existence of this mutant confirms the critical position of α-ketoglutarate on the crossroads of pathways of ammonia assimilation and of energy production.

MATERIALS AND METHODS

Bacterial strains. Bacteria used in this paper were *K. aerogenes* MK53 (*hutC515*), first described by Prival and Magasanik (7), and strain MK810, a mutant of strain MK53.

Cultivation of bacteria. Minimal medium was identical to that reported by Prival and Magasanik (7). Carbon sources were added at a final concentration of 0.4%. Nitrogen sources were added at 0.2% except where otherwise specified. Ammonium sulfate added at this concentration (equivalent to 30 mM) is designated high ammonia. Low ammonia is defined as ammonium sulfate at the concentration of 1 mM.

Isolation of strain MK810. An exponentially growing culture of strain MK53 was mutagenized with ethyl methane sulfonate for 90 min at 37 C. Subsequently, two identical penicillin enrichments were performed on an overnight culture of the mutagenized stock (9). The restrictive condition was glucose-high ammonia at 42 C, and the permissive condition was glucose-low ammonia at 30 C. Survivors were plated on glucose-low ammonia plates at 30 C. Individual colonies were scored at 30 and 42 C at both ammonia concentrations. Strain MK810 was unable to grow at 42 C in the presence of high ammonia but grew normally in the other conditions.

Chemicals. 3-Acetylpyridine nucleotide and coenzyme A (CoA) were obtained from PL Biochemicals. Other reagents have been described previously (7).

Enzyme assays. Total α-ketoglutarate dehydrogenase activity was measured as described by Kaufman et al. (3), except that extracts were dialyzed for no more than 24 h. Glutamine synthetase assays were performed on whole cells using cetyltrimethyl ammonium bromide as reported previously (7). Protein determinations were performed using the Folin phenol reagent (5).

RESULTS

Initial characterization. Strain MK810 was isolated as described above. Its growth on glucose minimal medium was normal at 30 C. At the restrictive temperature of 42 C, however, strain MK810 grew on glucose-low ammonia but not on glucose-high ammonia; it grew at the restrictive temperature with glutamate or

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histidine as sole source of nitrogen. In liquid culture, the mutant grew at growth rates comparable to those of the wild strain MK53 at all permissive conditions tested (Table 1). On solid high ammonia medium at 42 C growth was not visible for at least 2 days, with the exception that revertants appeared at a frequency of approximately 1 per 10^6 cells. Revertants were examined and determined to be wild type by all easily testable physiological criteria. Most probably, therefore, the mutant is a result of a lesion in a single site.

Shift experiments. Two types of shift experiments were performed to determine the nature of the ammonia effect. A temperature shift was done by growing cells in the presence of high ammonia at 30 C from a cell density of about 5 Klett units to 30 Klett units, upon which the cultures were shifted to 42 C. The ammonia shift experiment was done by growing the cells at 42 C with low ammonia until a cell density of 30 Klett units had been reached; ammonium sulfate was then added to bring the final concentration to 30 mM. The results are presented in Fig. 1. It can be seen that upon the shift to a restrictive condition, growth was significantly inhibited but did not stop immediately. Instead, it continued at a rapidly decreasing rate for about one generation before finally coming to a halt. The viable cell count remained constant for at least 3 h after the shift (data not shown).

The gradual nature of the growth shutoff in the shift experiments militated against the idea that failure of the mutant to inactivate glutamine synthetase by adenylylation should be the cause of the inhibition. Indeed, we found that the mutant rapidly adenylylates glutamine synthetase upon addition of 30 mM ammonium sulfate to a culture previously grown in low ammonia (11).

Further characterization of the growth response. Growth response tests were performed by individually adding a wide range of nutrients to glucose-high ammonia plates incubated at 42 C. The mutant grew on media supplemented with the following growth factors: sodium succinate, thiamine (vitamin B_1), methionine, cystathionine, homocysteine, β-alanine, pantothenic acid, pantoic acid, and pantetheine. Addition of L-alanine, L-serine, homoserine, or O-succinyl homoserine slightly stimulated the growth of the mutant. None of the many other amino acids, purines, pyrimidines, organic acids, or vitamins tested was effective.

Growth in liquid media determined the approximate effective concentration of added nutrient and the growth rate (Table 2). Methionine in low concentrations restored a normal growth rate. Low concentrations of the other growth-stimulating substances, with the exception of alanine, yielded a growth rate that was 50% slower. L-Alanine was effective in overcoming the ammonia effect only when added at the extremely high concentration of 20 mM. A level of 5 mM was completely ineffective.

The source of carbon used to grow strain MK810 was a significant parameter in regard to the phenotype. When grown with either citrate or pyruvate as sole source of carbon, the

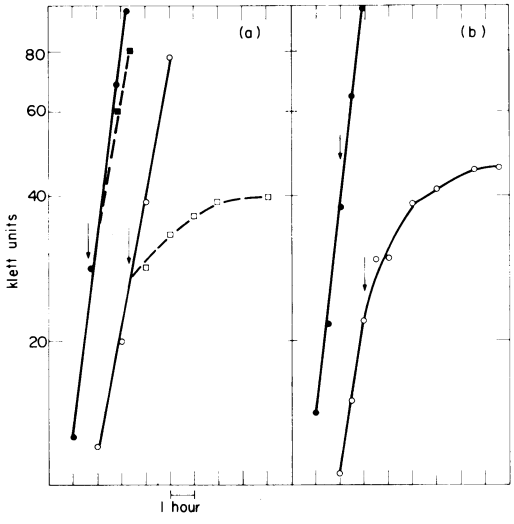

FIG. 1. *Effect of temperature and of ammonia on the growth of the wild-type strain MK53 and of the mutant strain MK810. (a) Cells of strain MK53 (●) and of strain MK810 (○) were grown at 30 C with high ammonia (30 mM). At the arrow, each culture was divided. One of each was allowed to continue at the same temperature; the other was shifted to 42 C (-----). (b) Cells of strain MK53 (●) and of strain MK810 (○) were grown at 42 C with low ammonia (1 mM). At the arrow, ammonium sulfate was added to bring the concentration to 30 mM.*

TABLE 1. *Growth of wild strain MK53 and mutant strain MK810 on different sources of nitrogen*

Nitrogen source	Temp (C)	Growth rate (generations/h)	
		Strain MK53	Strain MK810
NH_3 (30 mM)	30	1.3	1.3
NH_3 (30 mM)	42	1.5	NG[a]
NH_3 (1 mM)	30	1.0	1.1
NH_3 (1 mM)	42	1.4	1.3
Glutamate	30	0.2	0.2
Glutamate	42	0.5	0.5
Histidine	30	0.8	0.8
Histidine	42	0.8	0.8

[a] NG, No growth.

TABLE 2. *Nutritional requirements for growth of mutant strain MK810 on 30 mM NH_3 at 42 C*

Supplement	Growth rate (generations/h)
None	NG[a]
α-Ketoglutarate (1 mM)	NG
Succinate (2 mM)	0.7
L-Methionine (0.1 mM)	1.3
L-Methionine (1.0 mM)	1.3
L-Methionine (2.0 mM)	1.3
L-Alanine (5 mM)	NG
L-Alanine (20 mM)	0.7
β-Alanine (3 mM)	0.8
Pantothenate (1 mM)	0.9
Thiamine (2 μM)	0.9
Thiamine (20 μM)	0.9
Thiamine (50 μM)	0.9

[a] NG, No growth.

TABLE 3. *Temperature sensitivity of α-ketoglutarate dehydrogenase*

Strain[a]	Sp act at:	
	30 C	42 C
MK53	7.3	8.3
MK810	6.5	4.9

[a] The extracts were prepared from cells grown on citrate-high ammonia at 42 C.

[b] Activity is expressed as micromoles of α-ketoglutarate oxidized per minute per milligram of protein. The reactions were initiated by the addition of cell extract to the reaction mixtures maintained at the indicated temperatures. The numbers are the averages of duplicate determinations of the initial rates and agree within 10%. The experiment was repeated with a different pair of cell extracts and gave essentially the same results.

mutant is totally insensitive to inhibition by ammonia. When acetate was used as the sole source of carbon, there was a marked effect. At 30 C the mutant grew slowly when compared to the wild type, and not at all at 42 C. This effect occurred at all concentrations of ammonia. Addition of methionine in low concentrations overcame the inability of the mutant strain to grow on acetate at the high temperature.

Temperature sensitivity of α-ketoglutarate dehydrogenase. The substances which relieve the ammonia inhibition in glucose medium at 42 C fall into three classes: those that obviate the need for succinyl-CoA (the product of α-ketoglutarate dehydrogenase), those that are converted to succinyl-CoA, and those that are required cofactors for α-ketoglutarate dehydrogenase. These observations suggest that the lesion accounting for the mutant phenotype is in α-ketoglutarate dehydrogenase. Accordingly, dialyzed extracts of the wild and mutant strains grown on citrate-high ammonia at 42 C were assayed for α-ketoglutarate dehydrogenase at both 30 and 42 C. It can be seen from Table 3 that the mutant enzyme is noticeably temperature sensitive. At the high temperature, the mutant enzyme has less than 60% of the normal activity.

DISCUSSION

The physiological evidence reported above supports the view that the ammonia sensitivity of the mutant strain results from its inability to produce sufficient succinyl-CoA under the restrictive condition. The simplest explanation, accounting for all the diverse aspects of the phenotype, is a defect in α-ketoglutarate dehydrogenase. Preliminary biochemical tests indicate temperature sensitivity of the enzyme.

The growth deficiency at high temperature and high ammonia concentrations can be overcome by a variety of compounds falling into the following general classes: methionine and precursors of methionine biosynthesis; compounds such as succinate, which are used in alternative routes to succinyl-CoA; and precursors of certain required cofactors of α-ketoglutarate dehydrogenase, such as thiamine pyrophosphate and CoA. The various precursors to these compounds support the growth of the mutant, presumably because they either stabilize the enzyme complex or because the mutant enzyme has an altered affinity for them. Previously isolated mutants in *Escherichia coli* lacking this enzyme are able to grow in glucose minimal medium when both lysine and methionine are added (B. D. Davis, H. L. Kornberg, A. Nagler, P. Miller, and E. Mingioli, Fed. Proc. 18:211, 1959). The sole addition of methionine to a culture of strain MK810 is sufficient to overcome the inhibition exerted by ammonia, whereas addition of lysine is neither necessary nor sufficient. This may be explained by assuming that a lower internal level of succinyl-CoA is necessary for lysine biosynthesis, or that succinyl-CoA could be replaced by another activated compound. Aerobically grown bacteria using glucose as sole source of carbon are unable to obtain succinyl-CoA from fumarate by the action of succinate dehydrogenase; a different enzyme, repressed under aerobic conditions, is required for the conversion of fumarate to succinate (2). In fact, strain MK810 grows anaerobically at 42 C on glucose-high ammonia because it can apparently generate enough succinyl-CoA from fumarate (unpublished observation). The effect of L-alanine and L-serine added exogenously in abnormally high concentrations in allowing growth under restrictive

conditions may be explained by their inefficient conversion to β-alanine.

α-Ketoglutarate is at the branch point between carbon metabolism via the tricarboxylic acid cycle and nitrogen metabolism via the assimilation of ammonia into glutamate (Fig. 2). A competition for α-ketoglutarate therefore exists between α-ketoglutarate dehydrogenase and the enzymes responsible for the reductive amination of α-ketoglutarate to glutamate (10). We present the following hypothesis to account for the ammonia-sensitive phenotype of MK810. The mutant synthesizes a temperature-sensitive α-ketoglutarate dehydrogenase with an altered affinity for α-ketoglutarate at the restrictive temperature. At high ammonia concentrations, the defective protein is unable to compete with glutamic dehydrogenase for the limited quantity of α-ketoglutarate. Glutamic dehydrogenase is present at very high levels and is a rapid user of α-ketoglutarate. The K_m of glutamic dehydrogenase for α-ketoglutarate is lower by a factor of at least 10 than the K_m of α-ketoglutarate dehydrogenase (unpublished data). Growth on low ammonia alleviates the problem because glutamic dehydrogenase is not present (1). The problem may be alleviated in an analogous manner by addition of homoserine, which is known to be an inhibitor of glutamic dehydrogenase (4). The ammonia-sensitive phenotype is abolished when cells are grown on citrate or pyruvate, presumably because the intracellular concentration of Krebs cycle intermediates is higher. Why mutant cells are unable to grow on acetate at either concentration in the absence of methionine is not clear. Acetate may reduce the level of CoA and thus reduce the synthesis of succinyl-CoA by the defective α-ketoglutarate dehydrogenase.

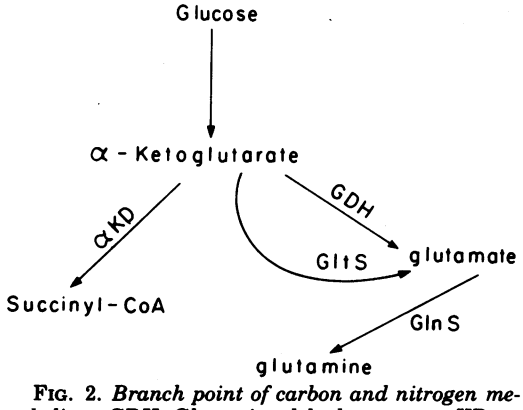

FIG. 2. *Branch point of carbon and nitrogen metabolism. GDH, Glutamine dehydrogenase; αKD, α-ketoglutarate dehydrogenase; GlnS, glutamine synthetase; GltS, glutamate synthase.*

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