Proc. Nat. Acad. Sci. USA
Vol. 72, No. 1, pp. 136–139, January 1975

Microbial Production of Ammonium Ion from Nitrogen

(N_2 fixation/nitrogenase/NH_4^+ excretion/*Klebsiella* mutants/*nif* regulation)

K. T. SHANMUGAM AND R. C. VALENTINE

Department of Chemistry, University of California, San Diego, La Jolla, Calif. 92037

Communicated by Martin D. Kamen, October 15, 1974

ABSTRACT **Genetic manipulation of nitrogenase and key glutamate-forming enzymes can provide mutants that excrete fixed N_2 as NH_4^+. A derepressed N_2 fixation mutant (SK-24) has been isolated, which excretes up to 20.2 μmol of fixed N_2 as NH_4^+ per mg of cell protein in 24 hr at room temperature. Biochemical analysis shows that this mutant, which requires glutamate for growth, releases fixed N_2 as NH_4^+ into the environment because of (*i*) constitutive synthesis of nitrogenase and (*ii*) genetic blocks resulting in losses of glutamate synthase [L-glutamine:2-oxoglutarate aminotransferase (NADPH oxidizing), EC 2.6.1.53] and glutamate dehydrogenase [L-glutamate:NADP oxidoreductase (deaminating), EC 1.4.1.4] activities, enzymes essential for NH_4^+ assimilation into cell material. The parent strain (*asm-1*), missing only glutamate synthase activity, also actively excretes NH_4^+ during early phases of its growth but eventually reutilizes the NH_4^+. A maximum yield of 4.0 μmol of NH_4^+/ml per 24 hr has been noted for *asm-1* only during the growth period. Biosynthesis of NH_4^+ proceeds at the expense of a variety of fermentable sugars, such as sucrose or glucose, with a maximum energy conversion efficiency of about 5 glucose degraded per NH_4^+ formed. The use of microbes for production of NH_4^+ fertilizer is discussed.**

There is now considerable experimental evidence that implicates glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] as a direct participant in genetic control of nitrogen fixation (*nif*). These findings can be summarized as follows: (*i*) We have found that glutamine-requiring auxotrophs (*glnA*$^-$) that lack catalytically active glutamine synthetase cannot synthesize nitrogenase (1). (*ii*) When *glnA*$^-$ mutants reacquire the glutamine synthetase (*gln*) genes as a result of conjugation with *Escherichia coli* carrying an F′ *gln* episome they can again make nitrogenase (2). (*iii*) *Klebsiella* mutants (GlnC$^-$) in which glutamine synthetase biosynthesis is constitutive continue to synthesize nitrogenase, in the presence of NH_4^+, at levels up to 30% of those produced in the absence of ammonia (1). (*iv*) Introduction of an F′ *nif* episome carrying the *nif* genes into GlnC$^-$ strains results in constitutive synthesis of nitrogenase (3). Recently we have constructed a *nif*-constitutive strain of *Klebsiella pneumoniae* (SK-24) which continues to synthesize nitrogenase, in the presence of NH_4^+, at levels up to 65% of those synthesized under derepressed conditions. Experiments reported here show that mutant SK-24 as well as its parent *asm-1* (4) excrete considerable quantities of fixed N_2 as NH_4^+. Some implications of these findings for commercial NH_4^+ production and as a model system for understanding NH_4^+ production by root nodule bacteria are discussed.

MATERIALS AND METHODS

Microbiological. The wild-type strain was *Klebsiella pneumoniae* M5A1. The isolation and properties of mutant *asm*-1 were as described previously (4). This mutant does not require exogenous glutamate or glutamate precursors, provided the medium contains greater than 1.5 mM NH_4^+ ions (4). Nitrogenase derepressed strain SK-24 was isolated as a glutamate auxotroph following treatment of *asm-1* for 30 min with 300 μg/ml of nitrosoguanidine (details for isolation of *nif* constitutive mutants will be presented in a separate publication). For growth on minimal medium (5) SK-24 must be supplemented with glutamate or a glutamate precursor such as aspartate, or glutamine.

NH_4^+ excretion experiments were performed at room temperature in 124-ml stationary flasks containing 50 ml of minimal medium which were constantly sparged with a stream of N_2 (or in certain instances argon) via glass tubes fitted through the stopper of the flask. Growth was measured by following the increase in optical density at 420 nm using a Gilford 300N spectrophotometer.

Biochemical. For NH_4^+ determination 0.1- to 0.3-ml aliquots of cell supernatant, clarified by centrifugal removal of the cells for 10 min at 12,000 × *g*, were added to micro-Conway diffusion chambers, which were incubated for 12 hr at room temperature to permit entrapment by diffusion of all the NH_4^+ in the center well. NH_4^+ was then determined colorimetrically using Nessler's reagent (6). Glucose was assayed by the ferricyanide procedure (7). Soluble protein was determined by the procedure of Lowry *et al.* (8) and whole cell protein by the method of Drews (9). Whole cell nitrogenase levels were measured using the acetylene method (10). Enzymatic activities of glutamate synthase [L-glutamine: 2-oxoglutarate aminotransferase (NADPH oxidizing), EC 2.6.1.53], glutamine synthetase, and glutamate dehydrogenase [L-glutamate:NADP oxidoreductase (deaminating), EC 1.4.1.4] were assayed using procedures described earlier (1). For determination of amino acids in the growth medium, aliquots clarified as above were added directly to the column of a Beckman model 121 automatic amino-acid analyzer for chromatography.

RESULTS

Biochemistry of NH_4^+-Excreting Strains. The patterns of NH_4^+ production from N_2 described in the next section were consistent with the levels of crucial enzymes synthesized by the NH_4^+-excreting strains (Table 1). For example, biochemical analysis revealed that strain SK-24 continued to synthesize nitrogenase, in the presence of NH_4^+, at levels up to 65% of those produced in the absence of NH_4^+. Note also that in this strain glutamine synthetase was produced constitutively (GlnC$^-$), whereas glutamate dehydrogenase activity was absent. As expected, this *nif* constitutive strain

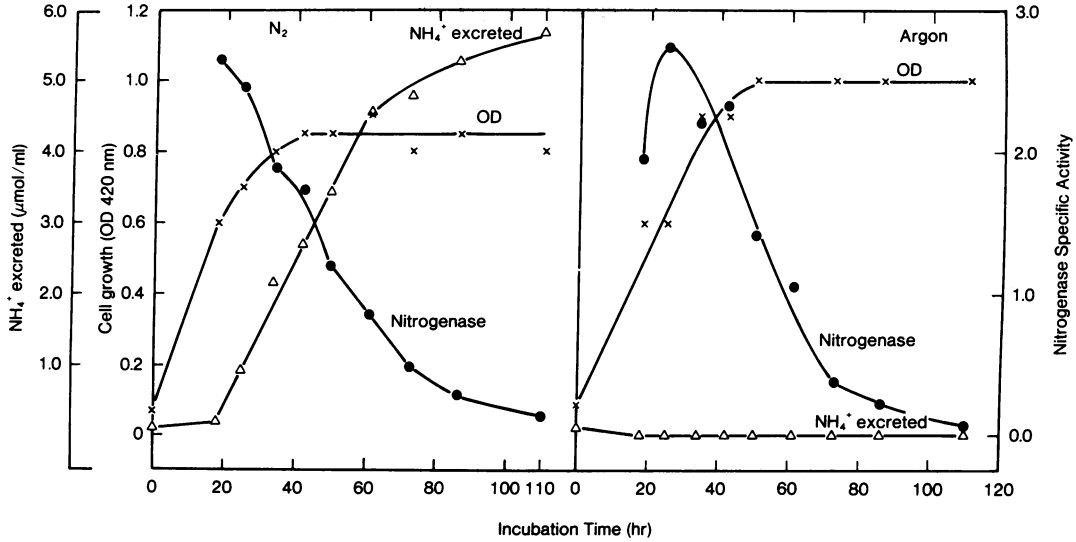

FIG. 1. Time course of NH_4^+ production from N_2 by derepressed *nif* strain SK-24. Details are given in the *text* and in *Materials and Methods*.

produced no glutamate synthase, a property shared with its parent *asm-1* (Table 1). Otherwise the enzyme levels of *asm-1* were similar to those of the wild type, i.e., in *asm-1* there was complete repression of nitrogenase by NH_4^+, several-fold repression of glutamine synthetase, and, conversely, strong induction of glutamate dehydrogenase in the presence of NH_4^+.

Patterns of NH_4^+ Production from N_2. The experiment summarized in Fig. 1 is divided into two parts; as shown in the left half of the figure, labeled "N_2", derepressed strain SK-24 was found to excrete up to 20.2 μmol of fixed N_2 as NH_4 per mg of cell protein during a 24-hr incubation period at room temperature with N_2 gas supplied by sparging. A maximum level of 5.6 μmol of NH_4^+ per ml of culture fluid was observed at the end of the experiment. Owing to the absence of both glutamate-forming enzymes this strain required supplementation with glutamate for growth, providing a convenient way to control cell yields during the experiment. From Fig. 1 it is seen that once growth ceased, production of NH_4^+ continued for several days. Exhaustion of glutamate needed for maintaining protein synthesis could explain the decrease in nitrogenase activity with consequent cessation of NH_4^+ production. An identical experiment was carried out as a control (Fig. 1—"Argon") except argon replaced N_2; of course, NH_4^+ was not produced in this case. Aliquots of the cell supernatant, removed at various times and analyzed using a Beckman automatic amino-acid analyzer, revealed only traces of amino acids other than the added glutamate, whose consumption was found to closely parallel that of cell growth. Most exogenous glutamate was utilized for cell growth prior to the peak period of NH_4^+ production, ruling out the possibility that this amino acid contributed its amino group for NH_4^+ production. Also, the maximum levels of NH_4^+ produced in this experiment far exceeded the total level of glutamate-NH_2. Thus, it seems probable that NH_4^+ itself was excreted into the supernatant, although the mechanism of transport of this ion across the cell membrane remained to be established.

Initial studies on NH_4^+ production by *asm-1*, parent of SK-24, where samples for NH_4^+ determination were taken only after several days' incubation, revealed some NH_4^+ production with values considerably lower than those of the constitutive strain SK-24. However, as summarized in Fig. 2b, closer inspection showed that NH_4^+ production by *asm-1* followed a biphasic curve, with NH_4^+ production dominant during the second day of incubation, followed by a decline in rates of NH_4^+ production best accounted for in terms of reutilization of the excreted NH_4^+. The evidence for this proposal was that cell growth continued for several days, well beyond exhaustion of added glutamate; thus growth must have occurred at the expense of excreted NH_4^+. Also *asm-1* has been reported to lose its requirement for glutamate, using NH_4^+ instead, at approximately the level of NH_4^+ encountered in this experiment (4). Two additional points are: (*i*) The maximum amount of NH_4^+ produced in a 24-hr period at room temperature with *asm-1* reached 4.0 μmol per ml of culture fluid—the highest values we have yet recorded for any of our mutants (see Fig. 2a); however, the rate of production on a cell protein basis was lower than for SK-24 (Fig. 2b). (*ii*) Other mutant

TABLE 1. *Enzyme levels of NH_4^+-producing strains*

Strain	Growth conditions	Glutamine synthetase	Glutamate synthase	Glutamate dehydrogenase	Nitrogenase (μmol/hr per mg of cell protein)
		(nmol/min per mg of protein)			
M5A1	$-NH_4^+$	492	12	<5	5.6
	$+NH_4^+$	167	31	101	0.0
asm-1	$-NH_4^+$	661	<5	18	2.5
	$+NH_4^+$	287	<5	194	0.0
SK-24	$-NH_4^+$	629	<5	<5	4.13
	$+NH_4^+$	759	<5	<5	2.70

Cells were grown anaerobically at 22° in sucrose minimal medium supplemented with 100 μg/ml of L-glutamate. When added, NH_4^+ was initially 15 mM. Cell-free extracts were prepared by disruption of cells with a French pressure cell as described previously (1). Enzyme assays were conducted as described in an earlier publication (1).

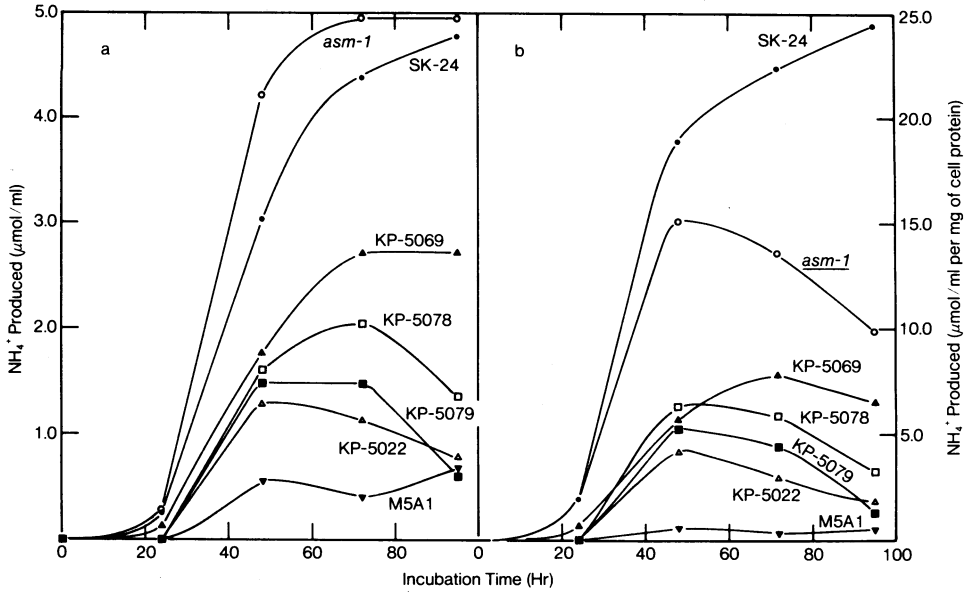

FIG. 2. Patterns of NH_4^+ production by different *Klebsiella* mutants. See *Materials and Methods* for experimental details. Properties and isolation of bacterial strains with designation KP 5069, etc. are summarized in Table 1 of ref. 1.

strains with altered glutamate or glutamine metabolism, such as *K. pneumoniae* K.P. 5069, are also capable of production of NH_4^+ in significant quantities, in contrast to the wild type, which excretes only traces of NH_4^+ (Fig. 2).

Energy Requirement for NH_4^+ Production. Nitrogenase is one of the most unusual enzymes known—the purified enzyme consumes as much as 15 moles of ATP per mole of N_2 substrate reduced to NH_4^+ (11, 12). In addition to ~P energy, nitrogenase also requires as substrate powerful reductants such as reduced ferredoxin, formation of which requires large numbers of additional ~P's. Nor is this a complete energy balance for fixation; one must add the ~P equivalents for synthesis of large amounts of the enzyme nitrogenase, etc.

As summarized in Table 2, a maximum energy conversion efficiency of about 5 glucose/NH_4^+ has been obtained with nongrowing cultures of SK-24 (for example between 48 and 60 hr of growth). For these calculations basal levels of glucose catabolism have not been deducted. This provides a means for an estimate of the moles of ATP utilized per mole of NH_4^+ produced, based on the assumption that SK-24 ferments glucose via the conventional glycolytic pathway to pyruvate. Since most coliform bacteria are able to extract additional ATP from pyruvate catabolism (13, 14), it is further assumed as a rough approximation that 1 additional ATP per glucose is obtained in this manner. Hence, one may suggest about 15 ATP/NH_4^+, so that N_2 fixation is an extremely costly energy-requiring process for the cell.

TABLE 2. *Glucose consumption by strain SK-24 during NH_4^+ production*

Age of the culture (hr)	Glucose consumed (μmol/ml)	NH_4^+ excreted (μmol/ml)	Glucose consumed/ NH_4^+ excreted*
0	0	0.09	
12	6	0.00	
24	12	0.78	7.69
36	17.8	2.00	4.75
48	23.8	3.23	4.88
60	29.7	4.42	4.96
72	35.7	4.98	10.71
84	41.6	5.23	23.60
96	47.5	5.45	26.82
108	53.5	5.63	33.33

Samples for glucose determination were taken during the experiment summarized in Fig. 1. NH_4^+ data are derived from Fig. 1.

* Ratio of μmol of glucose consumed per μmol of NH_4^+ excreted was calculated between successive time intervals.

DISCUSSION

Since wild-type cultures of *K. pneumoniae* excrete only traces of NH_4^+ into their environment during their growth cycle (Fig. 2), it appears that the NH_4^+ production and utilization under laboratory conditions must be closely balanc d systems, as one may expect for most biosynthetic processes. Indeed, the probable large energy output in terms of ATP and reducing power needed to support N_2 fixation may function selectively in imposing stringent conditions on growth if a tight regulation between N_2 fixation and the cellular consumption of NH_4^+ is to be maintained. However, such control is obviously not operational in agronomically important N_2-fixing organisms such as the symbiotic root nodule bacteria (bacteroids) of leguminous plants which excrete NH_4^+ for plant growth (15). It will be of considerable interest to pursue experimentally this analogy between NH_4^+-excreting *Klebsiella* and root nodule bacteria.

In *Klebsiella* it appears relatively easy by genetic manipulation to disrupt the tight integration between N_2 fixation and cell growth, thereby releasing fixed N_2 as NH_4^+ into the environment. The simplest procedure, as with *asm*$^-$ mutants, is to genetically interrupt the flow of NH_4^+ via glutamate into cell material without altering NH_4^+ production (nitrogenase activity). We envision the chain of events leading to NH_4^+ production by *asm*$^-$ strains as follows: (*i*) Nitrogenase is induced, catalyzing synthesis of NH_4^+. (*ii*) Transient auxotrophy resultant because the key glutamate-forming enzyme,

glutamate synthase, is missing (glutamate dehydrogenase, another enzyme transferring the NH_4^+N into glutamate, is repressed under these conditions) blocks conversion of this ammonia to glutamate. (*iii*) NH_4^+ produced by nitrogenase is transported in some unknown manner into the medium. (*iv*) As the level of NH_4^+ in the medium reaches a critical concentration (about 0.1 mM), glutamate dehydrogenase activity is expressed, providing a secondary pathway for reutilization of NH_4^+ for cell growth (abolishing glutamate auxotrophy). (*v*) As NH_4^+ accumulates in the medium, nitrogenase synthesis is repressed. We suggest that the mechanism of NH_4^+ production by the nitrogenase-derepressed strain SK-24 is similar to *asm-1* except that, firstly, the NH_4^+ reutilization step does not take place owing to continued repression of glutamic dehydrogenase and, secondly, nitrogenase is no longer subject to strong repression by NH_4^+. It is interesting to speculate that a $GlnC^-$ mutation may be responsible for the derepressed synthesis of nitrogenase and glutamine synthetase in strain SK-24, since the properties of this mutant resemble the $GlnC^-$ mutants described by Magasanik *et al.* (16). However, until the genetic lesion of SK-24 is mapped, other alternate explanations may be possible, such as a genetic defect blocking NH_4^+ assimilation to form glutamate in amounts sufficient to yield repressive levels of glutamine, a known repressor of nitrogenase (1).

It is of interest to note that Gordon and Brill (17) recently mentioned that addition of L-methionine-DL-sulfoximine (which apparently interferes with the ammonia assimilation), at a concentration of 28 mM, to cell suspensions of *Azotobacter vinelandii* results in the release of fixed ammonia.

Relatively large amounts of ATP (about 15 moles of ATP per mole of fixed N) may be consumed during N_2 fixation, a crucial fact to be remembered in proposals of microbial production of NH_4^+. Thus, the versatile blue-green algae might be ideal organisms for producing NH_4^+ fertilizer because these microbes utilize an unlimited energy source—solar energy—directly for N_2 fixation. Microbial production of NH_4^+ at the expense of other cheaply available energy sources such as cellulose or molasses might also prove feasible.

We are indebted to Dr. Martin D. Kamen for continued advice and encouragement. $GlnC^-$ strains of *K. pneumoniae* (K.P. 5069, 5078, 5079) were kindly supplied by Dr. S. L. Streicher. We thank Dr. C. Morandi for performing the glutamine synthetase assays. This work was supported by a grant from the National Science Foundation (GB-35331) and US Department of Agriculture Contract AG no. 12-14-1001-397. R.C.V. is the recipient of a U.S. Public Health Service Career Development Award (AI 16595).

1. Streicher, S. L., Shanmugam, K. T., Ausabel, F., Morandi, C. & Goldberg, R. B. (1974) *J. Bacteriol.*, in press.
2. Shanmugam, K. T., Streicher, S. L., Morandi, C., Ausabel, F., Goldberg, R. B. & Valentine, R. C. (1974) in *Symposium on Dinitrogen Fixation* (Kettering Foundation, Dayton, Ohio), in press.
3. Tubb, R. S. (1974) *Nature* **251**, 481–484.
4. Nagatani, H., Shimizu, M. & Valentine, R. C. (1971) *Arch. Mikrobiol.* **79**, 164–170.
5. Streicher, S. L., Gurney, E. & Valentine, R. C. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1174–1177.
6. Bodansky, O. (1971) in *Methods in Enzymology*, eds. Tabor, H. & Tabor, D. W. (Academic Press, New York), Vol. 17B, p. 889.
7. Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964) *Manometric Techniques* (Burgess Publ. Co., Minneapolis), p. 209.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
9. Drews, G. (1965) *Arch. Mikrobiol.* **51**, 186–198.
10. Shanmugam, K. T., Loo, A. & Valentine, R. C. (1974) *Biochim. Biophys. Acta* **338**, 545–553.
11. Ljones, T. & Burris, R. H. (1972) *Biochim. Biophys. Acta* **275**, 93–101.
12. Dalton, H. (1974) *Crit. Rev. Microbiol.* **3**, 183–220.
13. Knappe, J., Schacht, J., Möckel, W., Höpner, Th., Vetter, H., Jr. & Edenharder, R. (1969) *Eur. J. Biochem.* **11**, 316–327.
14. Krampitz, L. L. (1970) *Thiamine Diphosphate and Its Catalytic Functions* (Marcel Dekker, New York).
15. Dilworth, M. J. (1974) *Annu. Rev. Plant Physiol.* **25**, 81–114.
16. Magasanik, B., Prival, M. J., Brenchley, J. E., Tyler, B. M., DeLeo, A. B., Streicher, S. L., Bender, R. A. & Paris, C. G. (1974) in *Current Topics in Cellular Regulation*, eds. Horecker, B. L. & E. R. Stadtman, E. R. (Academic Press, New York), Vol. 8, pp. 119–138.
17. Gordon, J. K. & Brill, W. J. (1974) *Biochem. Biophys. Res. Commun.* **59**, 967–971.