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Regulation of Nitrogen Assimilation by the Obligate Chemolithotroph *Thiobacillus neapolitanus*

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The obligate chemolithotroph *Thiobacillus neapolitanus* can grow with NH_4^+, NO_3^- or urea as source of nitrogen. Gradual and rapid mechanisms were detected for regulating both the activity and the rate of synthesis of enzymes required for the metabolism of these nitrogen compounds. Glutamine synthetase (GS) in combination with glutamate synthase (GOGAT) was active under most growth conditions. Alanine dehydrogenase appears to be the major pathway of NH_4^+ assimilation during energy-limited growth in the presence of excess NH_4^+. GS was regulated in this organism by repression/derepression of enzyme synthesis, by inhibition by low molecular weight compounds, and also by adenylylation and deadenylylation. GS was deadenylylated during CO_2- and N-limited growth and also during energy-limited growth when NO_3^- or urea were supplied as the nitrogen source. GS was adenylylated during energy-limited growth in the presence of 7·7 mM-NH_4^+. The activity of GS increased with decreasing dilution rate during NH_4^+-limited growth, whereas the activity of GOGAT remained almost constant. The ability of whole cells to reduce NO_3^- was derepressed during N-limited growth. During NH_4^+-limited growth 24% of total carbon fixed was excreted as 2-oxoglutarate, pyruvate, succinate, *p*-hydroxyphenylacetate and ethylmalonate.

INTRODUCTION

Studies on Gram-negative bacteria, and in particular on the *Enterobacteriaceae*, have provided a detailed knowledge of nitrogen assimilation in these organisms. In general, glutamine and glutamate serve as the nitrogen donors for biosynthetic reactions in bacteria (for review see Tyler, 1978). If available in excess, ammonia is usually assimilated by the reductive amination of 2-oxoglutarate, catalysed by glutamate dehydrogenase (GDH) to give glutamate. Glutamate may then donate its amino group to a keto acid by transamination. In contrast, when the intracellular concentration of ammonia is relatively low, ammonia is usually assimilated by glutamine synthetase (GS), which, in an ATP-dependent reaction, adds an amino group to glutamate to give glutamine. A second enzyme, glutamate synthase (GOGAT), regenerates glutamate by a reaction involving glutamine and 2-oxoglutarate, whereby two molecules of glutamate are formed at the expense of NADPH. The GDH has a lower affinity for ammonia than the GS/GOGAT system. More energy is required to assimilate ammonia via the GS/GOGAT pathway than through GDH, so in many organisms GS is fully repressed and GDH is induced when excess ammonia is available. In cells grown in a medium containing a growth-limiting nitrogen source, synthesis of GS is derepressed and the enzyme is in its biosynthetically active form. Excess ammonia not only results in repression of GS but also in a direct feedback inhibition of the enzyme. Furthermore, the

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activity of GS can be decreased directly by a reversible adenylylation of the enzyme. Thus the biosynthetic activity of GS can be controlled in at least three ways. It is possible to measure the fully adenylylated, biosynthetically inactive enzyme in cell-free extracts by measuring its Mn^{2+}-dependent glutamyltransferase activity.

The complex regulation of ammonia assimilation occurs not only in *Enterobacteriaceae* (Tyler, 1978), but also in *Pseudomonas aeruginosa* (Janssen *et al.*, 1980), in N_2-fixing non-cyanobacteria (Kleinschmidt & Kleiner, 1978; Ludwig, 1978) and in photosynthetic non-cyanobacteria (Johansson & Gest, 1977). In N_2-fixing cyanobacteria the activity of GS seems to be regulated by conversion of an active form of the enzyme to an inactive form in response to variations of concentrations of divalent cations and reducing agents. This modulation of GS, which does not involve adenylylation, can be induced by darkness or by the addition of a high concentration of ammonia (Rowell *et al.*, 1979). GS of N_2-fixing cyanobacteria is also regulated by feedback inhibition (Stacey *et al.*, 1979).

Very little is known about the regulation of nitrogen assimilation in chemolithotrophic bacteria. Some *Thiobacillus* species which are unable to use exogenously supplied organic compounds as a source of energy are characterized by a lack of enzyme induction after addition of organic compounds to the growth medium. For this reason, various authors believe these organisms to be metabolically rigid (for reviews see Smith & Hoare, 1977; Matin, 1978). This raised the question as to how rigid these organisms would be with respect to changing inorganic environmental parameters like fluctuating concentrations and sources of nitrogen. This paper describes the regulation of nitrogen assimilation in the obligate chemolithotroph *Thiobacillus neapolitanus* during various growth conditions in the chemostat.

METHODS

Organism and growth conditions. Thiobacillus neapolitanus strain X was grown in the chemostat as described by Kuenen & Veldkamp (1973). The composition of the basal medium used in continuous cultivation for thiosulphate-limited growth of *T. neapolitanus* was the same as described previously (Beudeker *et al.*, 1980). This medium contained (%, w/v): NH_4Cl, 0·04; $MgSO_4.7H_2O$, 0·08; K_2HPO_4, 0·05; KH_2PO_4, 0·05; $Na_2S_2O_3.5H_2O$, 1·0; in deionized water plus 2 ml l^{-1} of a trace element solution (Vishniac & Santer, 1957). During thiosulphate-limited growth of *T. neapolitanus*, NH_4Cl was replaced, where indicated, by an amount of KNO_3 or urea giving the same nitrogen concentration. N-limited growth of *T. neapolitanus* was achieved by lowering the nitrogen content of the growth medium tenfold, for example to 0·004% (w/v) for NH_4^+-limited growth. Excess NH_4^+ was available for thiosulphate-limited cultures, because NH_4^+ was detectable in the supernatant and addition of more NH_4^+ did not result in the formation of more cells.

During all the above growth conditions, 1 M-Na_2CO_3 was used for neutralization of the sulphuric acid produced from the oxidation of thiosulphate. CO_2-limited growth of *T. neapolitanus* was obtained by removing CO_2 from the air by sodiumasbestos (Merck); a 50 mM-Na_2CO_3 solution was fed into the chemostat as growth-limiting substrate. NaOH (1·0 M) was used for titration during this growth condition. Details for CO_2-limited growth have been described by Kuenen & Veldkamp (1973). The N_2-fixing aerobic bacteria *Azotobacter vinelandii* strain O, *Beijerinckia indica* NCIB 8597 and *Xanthobacter flavum* 301 were kindly provided by Professor J. R. Postgate. These organisms were maintained as described by Strandburg & Wilson (1968) [for *X. flavum* as described by Biggins & Postgate (1969)]. Bacteria were harvested from steady-state chemostat cultures (unless described otherwise), and after washing the cells in the appropriate buffer, cell-free extracts were made according to Kuenen & Veldkamp (1973). Dithiothreitol (1 mM) was added to buffers.

Enzyme assays. Cell-free extracts were used to determine enzyme activities. Glutamine synthetase (GS; L-glutamate:ammonia ligase; EC 6.3.1.2) was assayed in three ways. (1) The biosynthetic activity (the ATP-dependent production of glutamine from glutamate and NH_4^+) was measured at 37 °C by following the release of inorganic phosphate from ATP as described by Shapiro & Stadtman (1970). In *Enterobacteriaceae*, only deadenylylated GS catalyses glutamine synthesis. (2) The Mn^{2+}-, ADP- and arsenate-dependent production of γ-glutamyl hydroxamate from glutamine and hydroxylamine (γ-glutamyltransferase activity) was taken as a measure of the total (adenylylated plus deadenylylated) GS activity. The procedure of Shapiro & Stadtman (1970) was followed. (3) The effect of Mg^{2+} on the γ-glutamyltransferase activity was determined. Addition of high concentrations of Mg^{2+} to cell-free extracts always resulted in lower GS transferase activity in cell free extracts of *T. neapolitanus*. In *Enterobacteriaceae* addition of high concentrations of $MgCl_2$ resulted in an increase of the

deadenylylated and a decrease of the adenylylated GS, respectively (Shapiro & Stadtman, 1970). The pH optimum for all three assays of GS was 7·3.

For snake venom phosphodiesterase (SVD) treatment of cell-free extracts, a dialysed cell-free extract (100 µl, containing 50 µg protein) was added to 1 ml 0·1 M-Tris/HCl, pH 8·8, containing 1 mM-$MgCl_2$. To this mixture 10 µl SVD (Boehringer) was added at 37 °C. At intervals of 30 min, 100 µl was assayed for GS biosynthetic activity (Ludwig, 1978).

Glutamate dehydrogenase (L-glutamate:NAD^+ oxidoreductase; EC 1.4.1.3) was assayed according to Schmidt (1974). Alanine dehydrogenase (L-alanine:NAD^+ oxidoreductase; EC 1.4.1.1) activity was determined at 37 °C following the procedure of Rowell & Stewart (1976). Glutamate synthase (GOGAT; L-glutamate:$NADP^+$ oxidoreductase; EC 1.4.1.13) activity was found to be optimal following a modification of the method of Lea & Miflin (1975). The assay mixture contained 50 mM-Tricine buffer, 5 mM-EDTA and 12·5 mM-mercaptoethanol adjusted to pH 7·8; 0·5 mM-2-oxoglutarate; 0·075 mM-NADPH; and cell-free extract (0·2 mg protein per ml of assay mixture). Glutamine (final concentration 5 mM) was added to start the reaction which was followed spectrophotometrically at 340 nm at 28 °C. Alanine aminotransferase (L-alanine:2-oxoglutarate aminotransferase; EC 2.6.1.2) activity was followed spectrophotometrically at 340 nm by coupling the production of pyruvate to NADH oxidation with lactate dehydrogenase according to Herbert *et al.* (1978).

NO_3^--reducing capacity of whole cells. An *in vivo* assay was used since no nitrate reductase activity was detected *in vitro*. Cells (0·1 mg protein) were washed once after centrifugation in a bench centrifuge in 0·1 M-phosphate buffer, pH 6·8. Propanol (5%, v/v) and NO_3^- (final concentration 10 mM) were added to a total volume of 1 ml. NO_2^- production was determined by adding 1 ml 1% (w/v) sulphanilamide in 1 M-HCl and 1 ml 0·01% (w/v) naphthylethylenediamine dihydrochloride after incubation at 28 °C for various times. After centrifugation for 15 min at room temperature in a bench centrifuge, the absorbance of the supernatant at 540 nm was read (Jaworski, 1971).

Characterization and quantification of the excreted products. Samples of steady-state culture fluids were filtered through a G5 glass filter, brought to pH 13 with Na_2CO_3, and freeze dried. Trimethylsilyl derivatives of the lyophilized residues were prepared according to Duran (1974). The derivatives were identified and assayed by gas–liquid chromatography according to Duran (1974).

RESULTS

Thiobacillus neapolitanus was able to use NH_4^+, NO_3^- and urea as nitrogen sources for growth. The yields of *T. neapolitanus* on NH_4^+, NO_3^- and urea during thiosulphate limitation in the chemostat were, respectively, 160, 117 and 132 mg dry wt l^{-1}. We were unable to grow *T. neapolitanus* on N_2 as nitrogen source, even at very low dissolved oxygen tensions (5% of air saturation). Although methylamine is a C_1 compound which can be used for growth by various methylotrophs (Meiberg, 1979), it was not used as a nitrogen source by *T. neapolitanus*.

Regulation of glutamine synthetase (GS)

Regulation of activity by small molecules. The biosynthetic activity decreased rapidly within 5 min of the addition of a high concentration of NH_4^+ to an NH_4^+-limited culture of *T. neapolitanus* (final concentration 7·7 mM-NH_4^+): this activity could be restored by dialysis (Table 1). Thus low molecular weight compounds had inhibited the GS biosynthetic activity. Dialysis of the extract of NH_4^+-limited bacteria harvested before the addition of excess NH_4^+ had no effect on the GS activity but dialysis of extract made after 5 min incubation with excess NH_4^+ fully restored the original activity. With increasing time the activity recovered by dialysis decreased, and became undetectable in the new steady state.

Regulation of glutamine synthetase activity by adenylylation. Dialysis restored only 10 to 20% of the original GS biosynthetic activity of extracts of bacteria harvested 1 h after the addition of excess NH_4^+ to a NH_4^+-limited culture (NH_4^+ shock) (Table 1). The GS transferase activity, however, had not decreased at that time, indicating that no enzyme proteolysis had occurred. Incubation of dialysed cell-free extracts with snake venom diesterase (SVD) resulted in complete restoration of the GS biosynthetic activity (Table 1). SVD is known to remove AMP residues from enzymes and thus it was concluded that GS activity in *T. neapolitanus* was also controlled by adenylylation/deadenylylation. No change was detected

Table 1. *Effect of NH_4^+ shock to a NH_4^+-limited culture of T. neapolitanus on GS biosynthetic and GS transferase activities and alanine dehydrogenase activity*

The dilution rate of the culture was 0·07 h^{-1} and the dissolved oxygen tension was 50% of air saturation. NH_4^+ was added to the culture (change-over) to give a final concentration of 7·7 mM. Extracts were assayed after dialysis and after snake venom diesterase (SVD) treatment where indicated.

Time after change-over (min)	Activity [nmol min^{-1} (mg protein)$^{-1}$]				
	GS biosynthetic	GS biosynthetic dialysed	GS transferase dialysed	GS biosynthetic SVD treated	Alanine dehydrogenase
0	215	200	1085	210	0
5	0	215	1098	220	1
60	0	36	1090	220	1
180	0	22	895	180	2
300	0	39	756	157	3
New steady state	0	0	100	19	21

Table 2. *Activities of various enzymes of nitrogen metabolism in T. neapolitanus grown under different limitations in the chemostat*

The dilution rate was 0·07 h^{-1} and the dissolved oxygen tension 50% of air saturation. The ability to reduce NO_3^- was assayed with whole cells; dialysed cell-free extracts were used for the other assays.

Limitation	N source	Activity [nmol min^{-1} (mg protein)$^{-1}$]						
		GS biosynthetic	GS transferase	Glutamate synthase	Alanine dehydrogenase	Glutamate dehydrogenase	Alanine aminotransferase	Capacity to reduce NO_3^-
Thiosulphate	NH_4^+	ND	100	127	21	2	14	–
Thiosulphate	NO_3^-	250	1033	194	ND	ND	NM	+
Thiosulphate	Urea	252	1060	152	ND	ND	NM	NM
Nitrogen	NH_4^+	215	1085	130	ND	ND	14	+
Nitrogen	NO_3^-	246	NM	238	ND	ND	NM	+
Nitrogen	Urea	370	2040	191	ND	ND	NM	+
CO_2	NH_4^+	50	210	161	ND	2	15	–
CO_2	NO_3^-	446	2700	154	ND	ND	NM	+

ND, Not detectable; NM, Not measured.

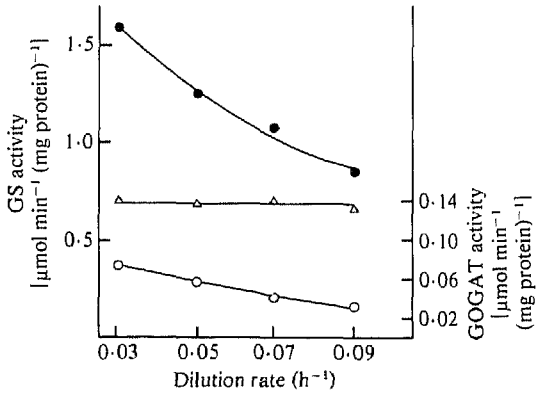

Fig. 1. The biosynthetic activity of glutamine synthetase (GS) (i.e. the ATP-dependent production of glutamine from glutamate and ammonia) (○), the transferase activity of GS (i.e. the production of γ-glutamylhydroxymate from glutamine and hydroxylamine) (●), and the activity of glutamate synthase (GOGAT) (△), in dialysed cell-free extracts of NH_4^+-limited *T. neapolitanus* cells as a function of dilution rate. The dissolved oxygen tension was 50% of air saturation.

in the pH optima of the GS biosynthetic and GS transferase activities as a consequence of adenylylation, in contrast to the findings of Janssen *et al.* (1980) for *Pseudomonas aeroginosa*. The GS biosynthetic activity was a nearly constant proportion (16 to 25%) of the GS transferase activity (Table 2), so during steady-state conditions either a constant or a relatively very small proportion of the total GS would be adenylylated. A pronounced shift in the extent of adenylylation of GS was, however, detected after the NH_4^+ shock (Table 1), until all GS was adenylylated. Treatment of whole cells with the stabilizing agent cetyltrimethylammonium bromide (Bender *et al.*, 1977) had no effect on GS biosynthetic activity (not shown). During thiosulphate-limited growth (7·7 mM-NH_4^+), GS was fully adenylylated, since no GS biosynthetic activity was detected, whereas GS transferase activity was measurable (Table 2). SVD treatment of the cell-free extract resulted in a GS biosynthetic activity of 19 nmol min^{-1} (mg protein)$^{-1}$ (Table 1), which again was about 20% of the GS transferase activity.

Regulation of the rate of synthesis of GS. Mn^{2+}-dependent GS transferase activity was detected in cell-free extracts of *T. neapolitanus* during all growth conditions (Table 2). As GS transferase activity is not subject to modification, activities of dialysed extracts provide an estimate of the quantity of GS protein. It is obvious from Table 2 that the GS transferase activity was dependent on the growth-limiting substrate, on the applied nitrogen source and also (Fig. 1) on the growth rate. In N-limited cultures, the GS transferase activity was derepressed about 10- to 20-fold compared with thiosulphate-limited cultures with excess NH_4^+ (7·7 mM). The derepression of the GS transferase is a result of the low intracellular NH_4^+ concentration rather than the physiological state of the cell (i.e. thiosulphate limitation or N limitation), since the GS transferase activity was also derepressed during thiosulphate limitation with excess NO_3^- or urea. During NH_4^+-limited growth, the GS transferase and the GS biosynthetic activity both increased with decreasing dilution rate (= growth rate) (Fig. 1). This is probably due to the decreasing concentration of the growth-limiting substrate (NH_4^+) with decreasing dilution rate.

Assimilation pathways of ammonia under various growth conditions

The derepression of GS during N-limited growth indicates that nitrogen is assimilated via GS to glutamine under these conditions. Glutamine can be metabolized to glutamate in an NADPH- and 2-oxoglutarate-dependent reaction catalysed by glutamate synthase (GOGAT). GOGAT activity varied only twofold, with maximal activity during NO_3^--limited growth and minimal activity during thiosulphate-limited growth with NH_4^+ as nitrogen source (Table 2). GOGAT activity appeared to be independent of the growth rate during NH_4^+-limited growth. During thiosulphate (energy)-limited growth with excess NH_4^+ as nitrogen source, no GS biosynthetic activity was detected because GS was completely adenylylated (see above). Under these conditions NH_4 assimilation is probably catalysed by alanine dehydrogenase (ADH), since this enzyme was sufficiently active to support the growth rate. Given a nitrogen content of 10% of the dry weight (unpublished results) it can be calculated that the required specific rate of nitrogen assimilation at a growth rate of 0·07 h^{-1} should be about 20 nmol min^{-1} (mg protein)$^{-1}$. Glutamate dehydrogenase (NADH- or NADPH-dependent) was insufficiently active to explain the growth rate during thiosulphate limitation and during CO_2 limitation with excess NH_4^+ (Table 2). The alanine formed by action of ADH might serve as an NH_4^+ donor in transaminase reactions. Activity of one enzyme catalysing such a reaction, L-alanine:2-oxoglutarate aminotransferase, was constant during all growth conditions tested (Table 2). ADH activity was induced slowly after the addition of excess NH_4^+ to an NH_4^+-limited culture (Table 1). Considering the low ADH activity shortly after the change-over, NH_4^+ assimilation would only partly be catalysed by ADH. Since the biosynthetic activity of GS was readily detected after dialysis (albeit decreased, due to partial adenylylation) NH_4^+ must be expected to be assimilated mainly via this enzyme until ADH is fully induced. Deadenylylated GS biosynthetic activity disappeared during this change-over at a rate equal to the dilution rate (Table 1).

Table 3. *Concentrations of the excretion products of T. neapolitanus during NH_4^+-limited growth in the chemostat*

The dissolved oxygen tension during growth was 50% of air saturation. The concentrations are expressed as mg C l^{-1}.

Dilution rate (h^{-1})	Total C excreted	Succinate	2-Oxoglutarate	Pyruvate	Ethylmalonate	*p*-Hydroxyphenyl-acetate
0·03	23	8	9	5	1	0
0·05	19	5	5	4	4	1
0·07	16	3	3	1	4	5

During CO_2-limited growth with excess NH_4^+, GS appeared not to by adenylylated and the activity had increased about twofold relative to a thiosulphate-limited culture with excess NH_4^+ (table 2). These results suggest that during CO_2 limitation, when excess energy is available, NH_4^+ is also assimilated through GS in *T. neapolitanus*.

NO_3^- assimilation in T. neapolitanus

NO_3^- assimilation proceeds through NO_2^- and NH_4^+ in bacteria in reactions catalysed by nitrate reductase and nitrite reductase, respectively (Dalton, 1979). Eight molecules of NAD(P)H have to be oxidized to reduce each NO_3^- to NH_4^+. In spite of numerous attempts, nitrate reductase activity was not detectable in cell-free extracts of *T. neapolitanus* grown on NO_3^- as a nitrogen source. An *in vivo* assay based on the detection of the NO_2^- formed was therefore used. Since part of the NO_2^- formed would be further reduced to NH_4^+, it appeared impossible to quantify the NO_3^--reducing activity. It was clear, however, that bacteria grown under N limitation (NH_4^+, NO_3^- or urea) were able to reduce NO_3^-, whereas those grown under thiosulphate limitation or CO_2 limitation with excess NH_4^+ were unable to reduce NO_3^- (Table 2). As expected, bacteria grown under thiosulphate or CO_2 limitation with excess NO_3^- were also able to reduce NO_3^- (Table 2). From these observations it was concluded that NO_3^--reducing capacity was derepressed during N-limited growth and repressed during thiosulphate or CO_2 limitation when no NO_3^- was available to the cells. NO_3^- *per se* is not necessary to derepress NO_3^- reductase.

Mutualistic interactions between T. neapolitanus and N_2-fixing heterotrophs

During NH_4^+-limited growth at dilution rates of 0·03, 0·05 and 0·07 h^{-1}, about 24% of total fixed carbon was excreted into the medium by *T. neapolitanus*. The excretion products were identified, by gas–liquid chromatography, to be 2-oxoglutarate, succinate, pyruvate, ethylmalonate and *p*-hydroxyphenylacetate. The concentrations of the individual excretion products were dependent on the growth rate (Table 3).

Microbial mutualism was reported to occur between *Thiobacillus ferrooxidans* and the N_2-fixing *Beijerinckia lacticogenes* (Tsuchiya *et al.*, 1974). It was suggested that *B. lacticogenes* would grow on the excretion products of the autotrophic *T. ferrooxidans* and that *T. ferrooxidans* would benefit from NH_4^+ excreted by *B. lacticogenes*. It was postulated that an analogous situation might occur if *T. neapolitanus* were to be grown in mixed cultures with N_2-fixing heterotrophs. Several N_2-fixing bacteria were tested for growth on supernatant fluid of NH_4^+-limited cultures of *T. neapolitanus*. *Xanthobacter flavum* was able to grow, while fixing N_2, on the *T. neapolitanus* supernatant, whereas *Beijerinckia indica*, *Azotobacter vinelandii* and *Klebsiella pneumoniae* did not grow. A mixed culture of *T. neapolitanus* and *X. flavum* (*D*, 0·05 h^{-1}; dissolved pO_2, 5% of air saturation) during NH_4^+ limitation with excess thiosulphate did not show significantly higher yields as compared with a pure culture of *T. neapolitanus* under the same growth conditions. The low oxygen tensions which were applied to minimize inhibition of nitrogenase by O_2 did not significantly influence the pattern of excretion products of *T. neapolitanus* in pure culture (not shown).

DISCUSSION

The obligate chemolithotroph *T. neapolitanus* appears to be very flexible with respect to the assimilation of nitrogen compounds. Glutamine synthetase plays a key role in nitrogen assimilation during N- and CO_2-limited growth, and also during energy-limited growth when NO_3^- or urea are applied as a nitrogen source. During energy-limited growth (7·7 mM NH_4^+), NH_4^+ assimilation is probably catalysed by alanine dehydrogenase, which was induced under these conditions (Table 2). Furthermore, alanine was the principally labelled amino acid during short-term [1-^{14}C]glycollate labelling studies carried out with thiosulphate-limited *T. neapolitanus* cells, but was not one of the early labelled products in NH_4^+-limited cells (Beudeker *et al.*, 1981).

Less energy is consumed when NH_4^+ assimilation is catalysed by alanine dehydrogenase than by the GS/GOGAT pathway. In a mutant of *Methylophilus methylotrophus* which possesses GDH instead of the wild-type GS/GOGAT, methanol yields are roughly 4 to 7% higher than in the wild type (Windass *et al.*, 1980). It should therefore be expected that the operation of the GS/GOGAT pathway in urea-grown *T. neapolitanus* cells should lead to lower yields during energy limitation. In fact the yields were more than 10% lower. As expected, yields of NO_3^--grown cells were even lower, because the reduction of nitrate requires reducing power in the form of NAD(P)H. In *T. neapolitanus*, reduced pyridine nucleotides must be formed directly through the oxidative pentose phosphate cycle or indirectly through the energy-consuming reversed electron transport. Alanine dehydrogenase has also been found to play a role in the assimilation of nitrogen in the cyanobacterium *Anabaena cylindrica* grown at high NH_4^+ concentrations (Rowell & Stewart, 1976).

The obligate chemolithotrophic thiobacilli show many physiological similarities with the obligate phototrophic cyanobacteria (for reviews see Smith & Hoare, 1977; Matin, 1978). GS in *T. neapolitanus* appears to be regulated not only by repression/derepression of enzyme synthesis and inhibition by low molecular weight compounds, but also by adenylylation control. No adenylylation system controlling GS activity has hitherto been reported for cyanobacteria (Rowell *et al.*, 1979; Stacey *et al.*, 1979). In *Escherichia coli*, and also in *Klebsiella aerogenes*, the average state of adenylylation of GS is determined by the ratio of 2-oxoglutarate to glutamine inside the cell (see review by Dalton, 1979). The excretion of 2-oxoglutarate by *T. neapolitanus* during N-limited growth (Table 3) is probably due to a high intracellular concentration of this compound. Thus the deadenylylation of GS during N-limited growth of *T. neapolitanus* might also be a consequence of the 2-oxoglutarate/glutamine ratio. Excretion of 2-oxoglutarate by obligate chemolithotrophic bacteria has also been reported by Pan & Umbreit (1972). These authors detected pyruvate, 2-oxoglutarate and *p*-hydroxyphenylpyruvate as excretion products during autotrophic growth in batch culture. It was suggested that these compounds might prevent heterotrophic growth on glucose of the obligate chemolithotrophic bacteria, since growth occurred in dialysis culture on glucose/salts media, in the absence of their specific inorganic energy source. Though heterotrophic growth of these obligate chemolithotrophic bacteria could not be shown by others (Matin, 1978) the physiological background for the excretion of the reported products remained an unsolved problem. A physiological explanation for the excretion of the keto acids 2-oxoglutarate and pyruvate by *T. neapolitanus* can now be given, since these compounds serve as acceptor molecules for NH_4^+ in transaminase reactions, and thus will accumulate during N limitation. Perhaps the batch cultures of Pan & Umbreit (1972) were N-limited. Metabolism of succinate (another excretion product of *T. neapolitanus* during N-limited growth: see Table 3) also depends on the presence of amino acids, since succinyl-CoA (directly derived from succinate) combines with glycine, in a reaction catalysed by 5-aminolaevulinate synthase, to form 2-amino-3-oxoadipate. It is suggested that lack of glycine results in intracellular accumulation of succinate and subsequently excretion. [Note that succinate is an end product of the tricarboxylic acid cycle in *T. neapolitanus*, since 2-oxoglutarate dehydrogenase is missing in this organism (see review by Matin, 1978)]. Pan

& Umbreit (1972), using thin-layer chromatography, detected *p*-hydroxyphenylpyruvate as an excretion product of *T. neapolitanus*, whereas we found *p*-hydroxyphenylacetate (identified by gas–liquid chromatography) as an excretion product of this bacterium. Intracellular accumulation of *p*-hydroxyphenylpyruvate (a precursor of tyrosine) due to a shortage of glutamate needed in the transaminase reaction, and subsequent decarboxylation to *p*-hydroxyphenylacetate may explain excretion of this compound by *T. neapolitanus*. At present no physiological explanation can be given for the excretion of ethylmalonate by *T. neapolitanus*.

The excretion of organic compounds by *T. neapolitanus* has ecological consequences. Heterotrophic bacteria will be able to grow in originally inorganic environments. Microbial mutualism between N_2-fixing bacteria and *T. neapolitanus*, as has been suggested to occur between *T. ferrooxidans* and *Beijerinckia indica* (Tsuchiya *et al.*, 1974) was not detected, however. This is possibly because *T. ferrooxidans* is itself able to fix N_2 (Mackintosh, 1978), whilst *T. neapolitanus* is not.

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