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Growth of *Hyphomicrobium* ZV620 in the Chemostat: Regulation of NH_4^+-assimilating Enzymes and Cellular Composition

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The regulation of the NH_4^+-assimilating enzymes glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT), and the cellular composition of *Hyphomicrobium* ZV620 grown in a chemostat with methanol and NH_4^+ as the supplied C- and N-sources were investigated. The influence of either C- or N-limitation (as a function of dilution rate) and of the C:N ratio (at a constant growth rate) was studied. $NADP^+$-dependent GDH was active at high NH_4^+-concentrations and was repressed at low NH_4^+-concentrations. The activity increased with increasing dilution rates under C-limited growth conditions. Derepression of $NADP^+$-dependent GDH was observed at low dilution rates under N-limited growth conditions. GS was more active at low NH_4^+-concentrations where both the total enzyme level (deadenylylated plus adenylylated forms) and the active fraction increased. C-limited growth resulted in low activities of GS, whereas activity in N-limited cells was consistently high. Dilution rate did not have a significant influence on the specific activity. The specific activity of GOGAT increased with decreasing NH_4^+ concentrations. Under both C- and N-limitation the specific activity of GOGAT increased with increasing growth rates. The C-content of the cells changed very little under the various growth conditions tested. The N-content and the protein content of the cells did not alter under C-limitation. N-limited growth conditions caused the cells to accumulate poly β-hydroxybutyrate. As a consequence, both the N-content and the protein content of the cells decreased.

INTRODUCTION

The development of interest both in methylotrophy and in the ecological significance of indigenous aquatic bacteria, particularly those bacteria capable of both suspended and attached growth, has resulted in marked expansion of knowledge concerning hyphomicrobia, not least the fact that under appropriate conditions they exhibit rapid and exponential growth (Wilkinson & Hamer, 1972), and that, in nature, they are frequently found in association with methanotrophic bacteria (Wilkinson *et al.*, 1974).

As carbon and energy substrates, many hyphomicrobia prefer C_1-compounds, particularly methanol, methylamine and formate, although some strains readily utilize multi-carbon compounds for growth. The oxidation and assimilation of methanol, together with the pertinent energy-yielding reactions, have been investigated and discussed (Harder & Attwood, 1978).

The ability of facultative, methylotrophic hyphomicrobia to denitrify is extensively documented (Sperl & Hoare, 1971; Uebayasi & Tonomura, 1976; Nurse, 1980), and it has been

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Abbreviations: D, dilution rate (h^{-1}); q_{CH_3OH}, specific methanol consumption rate [g methanol (g dry biomass)$^{-1}$ h^{-1}]; s_o, concentration of substrate in the inflowing medium; s, concentration of substrate in the culture liquid; $Y_{X/S}$, growth yield [g dry biomass produced (g substrate utilized)$^{-1}$]; GDH, glutamate dehydrogenase; AlaDH, alanine dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; PHB, poly β-hydroxybutyrate.

shown that these bacteria play an important role in both natural and technical denitrification processes. Although denitrification by some *Hyphomicrobium* spp. has been extensively investigated (Loginova *et al.*, 1976; Meiberg *et al.*, 1980) very little is known about either NH_4^+ or NO_3^--utilization by hyphomicrobia. In general, there are two pathways of major importance for NH_4^+-assimilation in bacteria. One pathway functions under conditions of excess NH_4^+ the other, under conditions of NH_4^+-deficiency. Both have been extensively discussed by Dalton (1979) and by Brown (1980).

The results of a study of the regulation of the NH_4^+-assimilating enzymes of *Hyphomicrobium* ZV620, growing in a chemostat with methanol as carbon energy substrate and NH_4^+ as N-source, under both methanol- and NH_4^+-limited conditions, are reported here. Data concerning the growth and cellular composition of, and methanol consumption by, *Hyphomicrobium* XV620 under various growth conditions are also presented.

METHODS

Organism and cultivation. Hyphomicrobium ZV620 was grown in chemostat culture in a bioreactor from MBR (Wetzikon, Switzerland) with a working volume of 1·65 l. The synthetic medium used was similar to that proposed by Bushnell & Haas (1941) and had the following composition: KH_2PO_4/Na_2HPO_4, 0·01 mol l^{-1}; $MgSO_4.7H_2O$, 0·20 g l^{-1}, $CaCl_2.2H_2O$, 0·020 g l^{-1}; $FeCl_2.4H_2O$, 3·0 mg l^{-1}; H_3BO_3, 0·75 mg l^{-1}; $ZnCl_2$, 0·10 mg l^{-1}; $MnCl_2.4H_2O$, 0·50 mg l^{-1}; $CuSO_4$, 0·050 mg l^{-1}; $CoCl_2.6H_2O$, 0·10 mg l^{-1}; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0·050 mg l^{-1}.

Before sterilization the pH of the medium was adjusted to 3·0 with conc. H_3PO_4. After sterilization at 121 °C, methanol and $FeCl_2$ were added to the cool medium after sterile filtration. In all experiments methanol and NH_4^+ were the sole sources of C and N, respectively, for the cells.

The temperature was maintained at 30 °C, and the pH was adjusted to 6·0 ± 0·05 by automatic addition of 2·0 M-KOH/NaOH (1:1, v/v). The stirring speed was 600 r.p.m. Dissolved O_2 tension was always kept higher than 10% saturation with air at 1 atm pressure.

Substrate concentrations. Methanol in the fermenter and the medium reservoir was analysed using a Shimadzu gas chromatograph (model RIA) equipped with flame ionization detector and a glass column (1·6 m × 4 mm) packed with Porapak QS (80–100 mesh). N_2 (60 ml min^{-1}) was the carrier gas and the column temperature was 165 °C. Ethanol was used as an internal standard. The detection limit for methanol was 1·0 mg l^{-1}. Parallel samples always showed less than ±4% deviation.

NH_4^+ was measured spectrophotometrically by an automatic $NH_4^+/NO_3^-/NO_2^-$-analyser system (Breda Scientific). Parallel samples showed less than ±1% deviation. The detection limit was 0·5 mg N l^{-1}.

Biomass. For the determination of biomass concentration cells were collected on a pre-weighed 0·4 μm Nuclepore polycarbonate filter. They were washed once with imidazole/HCl buffer (pH 7·0, 0·05 mol l^{-1}) and dried at 105 °C to constant weight. Parallel samples varied by less than ±2%.

Elemental and cellular composition. For the determination of C, N and poly β-hydroxybutyrate (PHB) the cells were separated by centrifugation, washed once with imidazole/HCl buffer (pH 7·0, 0·05 mol l^{-1}) and freeze-dried. After subsequent drying over P_2O_5 in a desiccator, the C- and N-contents of the freeze-dried cells were measured with a F&M Scientific model 185 C, H, N-Analyzer. PHB was estimated according to the modified method of Karr *et al.* (1983). To obtain partially purified PHB, approximately 0·1 g of the freeze-dried cells were digested in 1 ml of approximately 14% sodium hypochlorite for 2 h at 40 °C. After adding 9 ml distilled water the remaining suspended material was separated by centrifugation (30000 ***g*** for 10 min), washed with water and then with ethanol, and subsequently dried for 30 min at 100 °C. PHB contained in this dried cell material was suspended in 1 ml conc. H_2SO_4 and heated for 30 min at 90 °C to yield crotonic acid. After dilution (1:1000) with distilled water, crotonic acid was determined by using a gas chromatograph as described by Karr *et al.* (1983), except that the column temperature used was 140 °C. The H_2SO_4 left after dilution (0·02 M) did not markedly influence the determination.

For the determination of protein, cells from 5 ml of culture fluid were separated by centrifugation, washed once with buffer as described above, and suspended in 2 ml distilled water. The biuret method was used to measure protein. Bovine serum albumin (Sigma) was used as a standard.

Preparation of cell-free extracts. Cells (100–200 mg dry wt) were separated by centrifugation (30000 ***g***, 5 min) at 4 °C and washed twice with ice-cold imidazole/HCl buffer (pH 7·0, 0·05 mol l^{-1}). The resulting pellet was resuspended in 10 ml of the same buffer and cell breakage was achieved by passing them twice (at 85 MPa) through a French pressure cell (Aminco D4 3398A). Whole cells and cell debris were removed by centrifugation at 4 °C, (20000 ***g***, 20 min) and the resulting supernatant, which contained 4–8 mg protein ml^{-1}, was then assayed for all the enzymes of interest. Protein in the cell-free extract was measured by the biuret method.

Enzyme assays. All enzyme assays were done at 30 °C using a Unicam SP 1800 UV spectrophotometer. Glutamate dehydrogenase (GDH, L-glutamate:NAD(P)$^+$ oxidoreductase, EC 1.4.1.3) was assayed for NAD$^+$- and NADP$^+$-dependent activity. The assay mixture contained KH_2PO_4/K_2HPO_4 buffer (pH 7·8, 0·1 mol l^{-1}); 2-oxoglutarate, 0·01 mol l^{-1}; NAD(P)H, 0·16 mmol l^{-1} and NH_4Cl, 0·1 mol l^{-1}. The reaction was started by addition of NH_4Cl. Using cell-free extract the K_m of NADP$^+$-dependent GDH from *Hyphomicrobium* ZV620 for NH_4^+ was in the range 8·6–9·4 mM (Eadie–Hofstee, Hanes or Lineweaver–Burk plots).

Alanine dehydrogenase (AlaDH, L-alanine:NAD$^+$ oxidoreductase, EC 1.4.1.1) was assayed as was GDH except that 2-oxoglutarate and NAD(P)H were replaced by pyruvate and NADH, respectively.

Glutamate synthase (GOGAT, L-glutamine:2-oxoglutarate aminotransferase, EC 1.4.1.14) was assayed as was GDH, except that NH_4Cl was replaced by glutamine (0·01 mol l^{-1}) and only NADH (0·16 mmol l^{-1}) was used. The reaction had to be started with cell-free extract, otherwise complete loss of activity was observed. This effect was reported also for GOGAT from *Klebsiella pneumoniae* (Brenchley *et al.*, 1973). GOGAT from *Hyphomicrobium* ZV620 was NADH-dependent only and no activity with NADPH was detected.

Glutamine synthetase (GS, L-glutamate:ammonia ligase, EC 6.3.1.2.) activity was measured in two ways. Biosynthetic activity was assayed as described by Murell & Dalton (1983). γ-Glutamyl transferase activity (adenylylated plus deadenylylated form) was assayed using the method first described by Shapiro & Stadtman (1970) which was modified in the following way: imidazole/HCl buffer, pH 6·9, 0·04 mol l^{-1}; $MnCl_2$, 0·04 mol l^{-1}; (NH_4OH)Cl + NaOH, 0·06 mol l^{-1}; potassium arsenate, 0·1 mol l^{-1}; ADP, 0·4 mmol l^{-1}; glutamine, 0·03 mol l^{-1}. The reaction was started by the addition of glutamine. Transferase activity of the active (deadenylylated) form of GS only was measured by the addition of $MgCl_2$ (0·06 mol l^{-1}) to the assay described above. Activity ratios (GS:GS-AMP + GS) were calculated on the basis of the two transferase assays. The isoactivity point of the two forms of GS was measured according to the method described by Bender *et al.* (1977). Using cell-free extract the isoactivity point was 6·9: hence all measurements were carried out at this pH.

Enzyme units. The specific activities of all enzymes are expressed as the rate of substrate consumption (or product formation) per unit of protein [μmol min^{-1} (mg protein)$^{-1}$].

RESULTS

Effect of the C:N ratio at a fixed dilution rate

Hyphomicrobium ZV620 was grown in a chemostat at a constant dilution rate of 0·054 h^{-1}. The concentration of the N-source was kept constant in the medium reservoir [$s_o(NH_4^+\text{-N})$ = 223 mg l^{-1}]. To achieve different C:N ratios (given as g C per g N) in the growth medium the concentration of methanol in the reservoir was increased step by step from s_o (CH_3OH) = 0·95 g l^{-1} (C:N = 1·60) to 12·4 g l^{-1} (C:N = 20·85). Based on substrate concentrations, cell composition and synthesis of NH_4^+-assimilating enzymes, three distinctly different growth regimes can be recognized: methanol-limited, transition and NH_4^+-limited.

Growth and cellular composition. The concentration of the residual substrates, i.e. methanol and NH_4^+, and the biomass produced in the course of the experiment described above, are shown in Fig. 1(*a*). Increasing the C:N ratio in the inflowing medium resulted in a linear increase of the biomass concentration in the culture. This was the case both when methanol was the growth-limiting substrate and during the transition growth regime. When medium with a C:N ratio >12·6 was supplied, residual methanol became detectable in the culture and the growth yield with respect to methanol dropped from Y_{X/CH_3OH} = 0·38 ± 0·02 (for C:N ≤12·6) to Y_{X/CH_3OH} = 0·29 (at C:N = 20·9). During C-limited growth the cells synthesized no PHB, but PHB started to accumulate within the cells as soon as residual NH_4^+ could no longer be detected in the culture (Fig. 1*b*). This accumulation of PHB explains the further increase of the biomass concentration under transition and NH_4^+-limited growth conditions as the PHB deposited in the cells accounts for between 70% (at C:N = 13·1) and 90% (at C:N = 21·3) of the surplus biomass produced under such growth conditions. The protein, C- and N-contents of the cells are shown in Fig. 1(*b*). In cells grown under N-limitation, the protein content and N-content were reduced to approximately 65–75% of that in cells grown under C-limitation. Although the cells accumulated PHB under N-limited growth conditions (average C-content = 56%) the cellular C-content did not increase significantly, a feature which can be explained by the concurrent reduction in protein content.

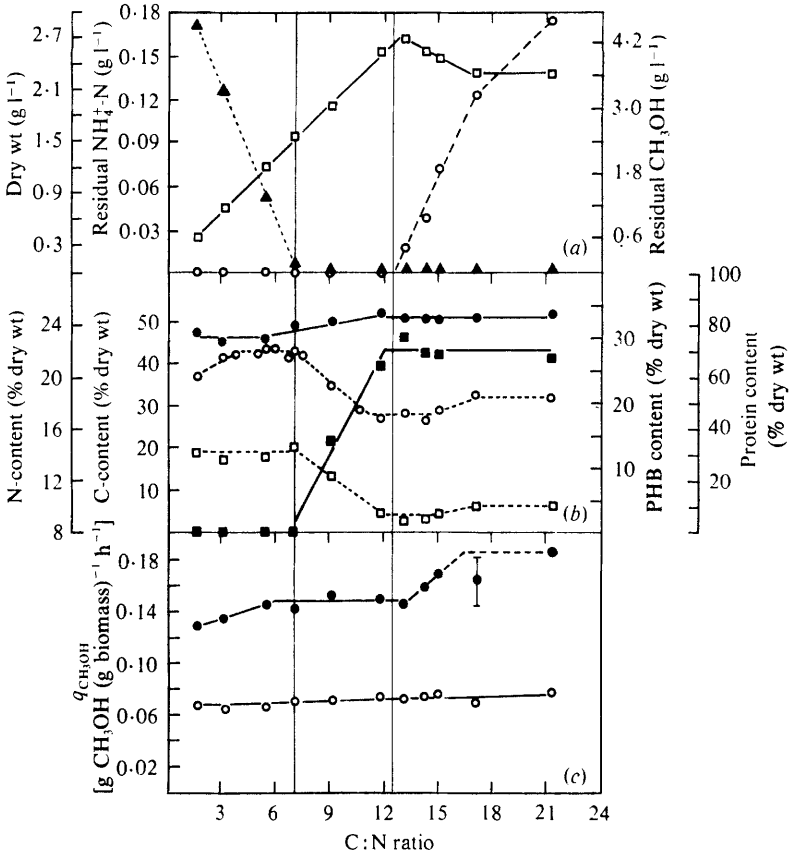

Fig. 1. Growth of *Hyphomicrobium* ZV620 with methanol and NH_4^+ in the chemostat at a constant dilution rate (D = 0·054 h^{-1}), as a function of the C : N ratio in the feed. (*a*) Dry wt of biomass produced (□), and residual concentrations of methanol (○) and NH_4^+-N (▲). (*b*) Cellular content (percentage dry wt) of C (●), N (□), protein (○) and PHB (■). (*c*) Specific rates of total methanol consumption by the cells (●) and specific rate of methanol consumption used for assimilation (○). The vertical line at C : N = 7·1 indicates the transition to N-limited growth conditions; the line at C : N = 12·6 indicates the occurrence of residual methanol in the culture.

A distinct increase in the specific rate of methanol comsumption, q_{CH_3OH}, was observed for cells growing in media with C : N ratios >13·1 (Fig. 1*c*). The fact that the specific rate of C-assimilation, calculated from the C-content of the cells, was constant over the whole range of C : N ratios tested, indicates that more carbon from methanol is channelled into dissimilatory processes during N-limited growth.

Enzyme specific activities. As found for many other bacteria $NADP^+$-dependent GDH was active in cells of *Hyphomicrobium* ZV620 NH_4^+ when was present in excess, whereas the high-affinity (GS/GOGAT) NH_4^+-assimilation pathway, was responsible for the N-assimilation when the residual concentration of NH_4^+ in the culture was low (Fig. 2*a–d*). Both NAD^+- and $NADP^+$-dependent specific activity responded to changes of the NH_4^+ concentration in the environment, the specific activity of NAD^+-dependent GDH showed no dependency on any of the parameters studied, and varied within the range 0·005–0·015 μmol min^{-1} (mg protein)$^{-1}$. This suggests that *Hyphomicrobium* ZV620 synthesizes two different GDHs, of which the $NADP^+$-dependent form is involved in the assimilation of NH_4^+, whereas the function of NAD^+-dependent GDH is probably only catabolic (see, e.g. Dalton 1979). In order to

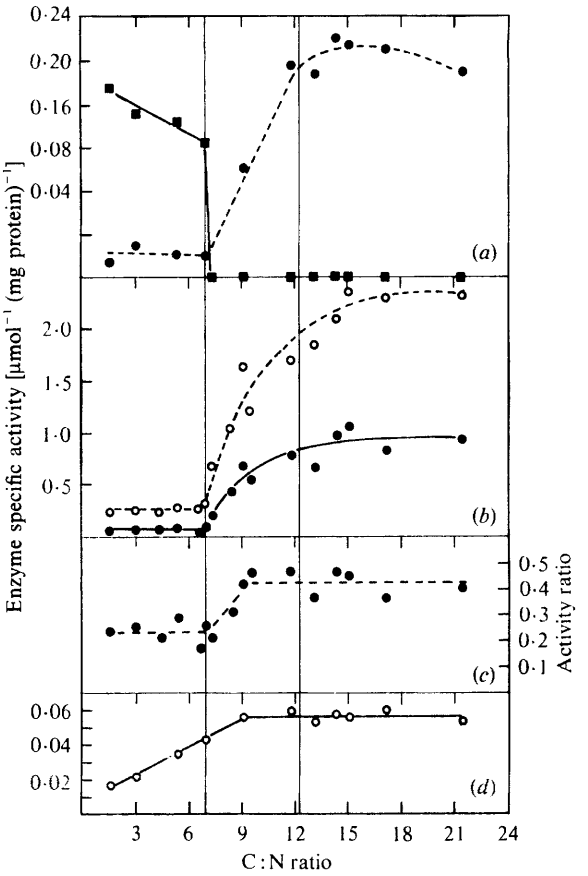

Fig. 2. Activities of enzymes involved in the assimilation of NH‡ in cell-free extracts of *Hyphomicrobium* ZV620 grown with methanol and NH‡ in a chemostat at a constant dilution rate (D = 0·054 h^{-1}), as a function of the C:N ratio of the inflowing medium. (*a*) Specific activities of $NADP^+$-dependent GDH (■) and GS (●, biosynthetic activity). (*b*) Specific transferase activities of GS: ○, adenylylated plus deadenylylated form; ●, deadenylylated form only. (*c*) GS transferase activity ratio (deadenylylated GS activity : adenylylated plus deadenylylated GS activity). (*d*) Specific activity of GOGAT. For the meaning of vertical lines at C:N = 7·1 and 12·6 see Fig. 1.

differentiate between total GS protein synthesized (adenylylated plus deadenylylated form) and the enzymically active fraction of GS protein (deadenylylated form) the transferase reaction assay was used (Fig. 2*b*, *c*). It can be concluded from the specific transferase activities that the change from C- to N-limited growth increased the concentration both of total GS protein in the cells and also the fraction of active GS. In cells grown under methanol limitation only about 20% of the GS was deadenylylated (GS : GS-AMP + GS ≃ 0·21) whereas nearly 50% of the GS was deadenylylated under N-limited growth conditions (GS : GS-AMP + GS ≃ 0·45). In contrast to GS, no clear onset of derepression, induced by the low extracellular NH‡-concentration, was found for GOGAT (Fig. 2*d*). The specific activity of this enzyme increased linearly from 0·017 (C:N = 1·6) to 0·057 μmol min^{-1} (mg protein)$^{-1}$ (C:N = 9·1) with increasing C:N ratios in the feed. A further increase of the C:N ratio had no influence on the specific activity of GOGAT. No significant activity of AlaDH was detected in cell-free extracts of *Hyphomicrobium* ZV620. Irrespective of the growth conditions, its specific activity was always approximately 0·006 μmol min^{-1} (mg protein)$^{-1}$.

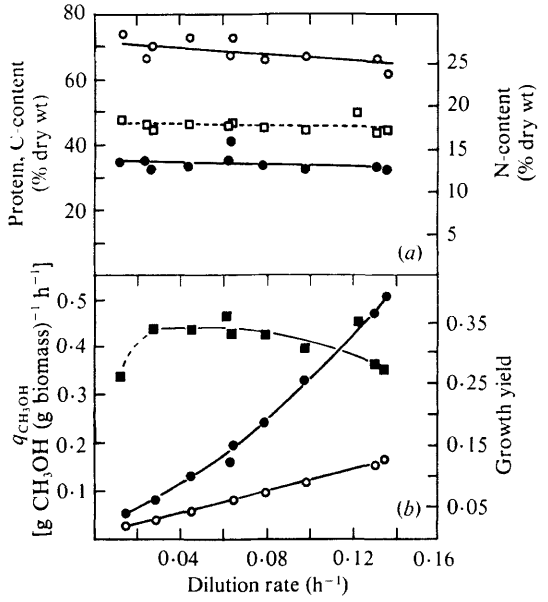

Fig. 3. Growth of *Hyphomicrobium* ZV620 in methanol-limited chemostat culture. (*a*) Cellular content of C (□), N (●) and protein (○). (*b*) Specific rate of total methanol consumption (●), specific rate of methanol consumption used for assimilation (○) and growth yield for methanol (■).

Effect of dilution rate on methanol-limited growth

Growth and cellular composition. Hyphomicrobium ZV620 was grown in a chemostat with methanol as the growth-limiting substrate. The C : N ratio of the growth medium in the reservoir was 6·0 [4·05 g l^{-1} ≤ $s_o(CH_3OH)$ ≤ 4·55 g l^{-1}; 0·26 g l^{-1} ≤ $s_o(NH_4^+\text{-N})$ ≤ 0·28 g l^{-1}]. The resulting residual NH_4^+-N concentration in the culture was in the range 0·07–0·10 g l^{-1}. The critical dilution rate for the culture was 0·14 h^{-1}. The C- (45 ± 2%) and N- (12·9 ± 0·3%) contents of the cells were constant over the whole range of dilution rates investigated (Fig. 3*a*). The protein content of the cells decreased linearly with increasing dilution rate from 73 to 64% (Fig. 3 *a*). This was probably due to an increasing demand for RNA at higher growth rates (Herbert, 1961). The cells did not accumulate PHB at any of the dilution rates tested. The specific rate of methanol consumption (q_{CH_3OH}) increased linearly up to a dilution rate of approximately 0·08 h^{-1}. At higher dilution rates q_{CH_3OH} increased overproportionally (Fig. 3*b*). However, the specific rate of carbon (q_C) assimilated increased linearly with the growth rate. This explains the drop in growth yield from 0·34–0·36 g (g methanol)$^{-1}$ to 0·27 g (g methanol)$^{-1}$ at higher growth rates (D = 0·136 h^{-1}).

Enzyme specific activities. The specific activities of $NADP^+$-dependent GDH in cell-free extracts of *Hyphomicrobium* ZV620 grown under methanol-limitation increased with increasing growth rates as shown in Fig. 4*b*. As expected for these growth conditions the specific activity of GS was low at all dilution rates (Fig. 4*a*, *b*). Additionally, the GS : GS-AMP + GS ratio was in the range 0·08–0·19, i.e. almost all of the GS protein was present in its inactive form. The specific activity of GOGAT was almost constant at 0·04 μmol min^{-1} (mg protein)$^{-1}$ except for dilution rates below D = 0·04 h^{-1} (Fig. 4*b*).

Effect of dilution rate on NH_4^+-limited growth

Whereas no problems were encountered during the growth of *Hyphomicrobium* ZV620 under methanol-limited growth conditions in the chemostat, several difficulties arose when the bacterium was grown under NH_4^+-limitation. Thus, at high dilution rates, the cells were sensitive to residual methanol, and wash-out, wall growth and the formation of small, misshapen cells was observed. However, under transition growth conditions none of these adverse

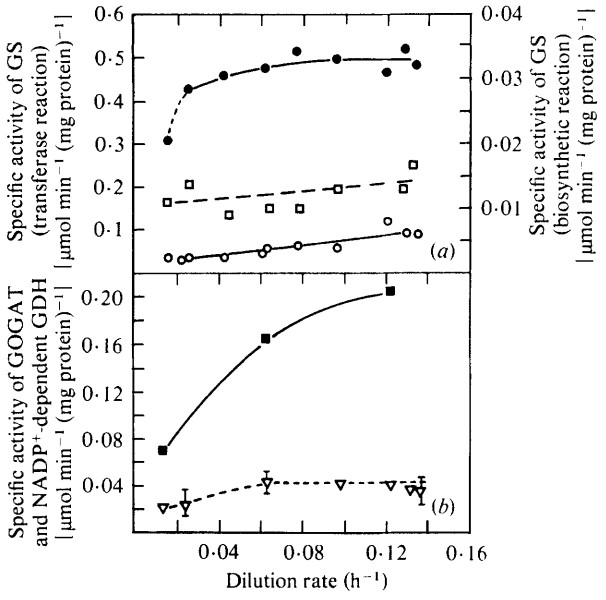

Fig. 4. Activities of enzymes involved in NH‡-assimilation in cell-free extracts of *Hyphomicrobium* ZV620 grown under methanol-limitation in a chemostat. (*a*) Specific activities of GS catalysing the biosynthetic reaction (□) and the transferase reaction (●, adenylylated plus deadenylylated form; ○, deadenylylated form only). (*b*) Specific activities of NADP⁺-dependent GDH (■) and GOGAT (▽).

effects were noticeable. Because the cells also exhibited an N-limited phenotype (i.e., GS/GOGAT expressed, GDH repressed, PHB synthesized) it was decided to study the influence of the growth rate on 'N-limited' cell physiology and composition under transient conditions rather than under N-limited growth conditions. Therefore cells were grown on a medium with a C : N ratio of 9·5 [$s_0(CH_3OH)$ = 5·50 g l^{-1}, $s_0(NH‡\text{-N})$ = 0·23 g l^{-1}]. However, as the C-requirement (and probably also the N-requirement) was also dependent on growth rate, the C : N ratio of the inflowing medium had to be increased slightly to 11·8 at D = 0·01 h^{-1} in order to avoid NH‡-accumulation in the culture.

Growth and cellular composition. Over the whole range of dilution rates tested (0·010 $h^{-1} \leq D \leq$ 0·101 h^{-1}), the C-content of the cells was constant (49·0 ± 1·5%), whereas the N-content and protein content decreased with increasing growth rates from 12·0 to 10·5% and from 62 to 49%, respectively. At all dilution rates the cells accumulated PHB. It was somewhat surprising to find that the PHB content of the cells was only approximately 10% at low dilution rates and increased to 21–23% at $D < 0{\cdot}06$ h^{-1}. The low PHB content at D = 0·01 h^{-1} is not simply a result of the higher C : N ratio in the medium supplied to the cells at this growth rate (C : N = 11·8), because at D = 0·04 h^{-1} (when the C : N ratio of the medium used was 9·5) the cellular content of PHB was also found to be considerably lower.

Enzyme specific activities. As expected, high specific activities of GS and GOGAT were detected in cell-free extracts of 'NH‡-limited' cells. No clear dependency of the specific enzyme activity for either the biosynthetic or the transferase reactions was observed (Fig. 5*a*). Approximately 30% of the GS was deadenylylated at all dilution rates. However, the specific activity of GOGAT increased almost linearly with increasing growth rates (Fig. 5*b*). At dilution rates lower than D = 0·04 h^{-1} there was increasing derepression with decreasing growth rates of the specific activity of NADP⁺-dependent GDH (Fig. 5*b*). At D = 0·01 h^{-1} the specific activity of NADP⁺-dependent GDH in 'N-limited' cells reached approximately 20% of the specific activity found for this enzyme in cells grown under C-limitation at the same growth rate.

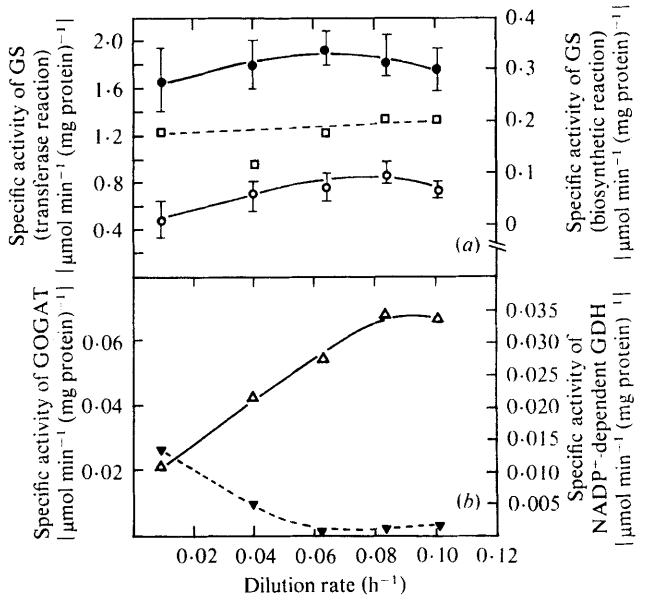

Fig. 5. Activities of enzymes involved in NH_4^+-assimilation in cell-free extracts of *Hyphomicrobium* ZV620 grown in NH_4^+-limited chemostat culture. (*a*) Specific activities of GS catalysing the biosynthetic (□) and the transferase reaction (●, adenylylated plus deadenylylated form; ○, deadenylylated form only). (*b*) Specific activities of $NADP^+$-dependent GDH (▼) and GOGAT (△).

Table 1. *Influence of the N-source on specific activities of N-assimilating enzymes in cell-free extracts of Hyphomicrobium ZV620*

Hyphomicrobium ZV620 was grown in a chemostat (D = 0·55 h^{-1}) under either C(methanol)- or N-limitation.

Growth regime	N-source	Enzyme specific activity [μmol min^{-1} (mg protein)$^{-1}$] NADP⁺-dependent GDH	GS (biosynthetic activity)	GOGAT
C(methanol)-limitation	NH_4^+	0·131	0·021	0·041
	NO_3^-	<0·001	0·070	0·037
N-limitation	NH_4^+	<0·001	0·202	0·057
	NO_3^-	<0·001	0·110	0·045

Effect of NO_3^- as the N-source

Hyphomicrobium ZV620 can also grow with NO_3^- as the sole N-source. No uniformity seems to exist amongst microbes with respect to the enzymes by which NH_4^+ produced from NO_3^- (after reduction via the assimilatory NO_3^- reductase and NO_2^-reductase) is assimilated since operation of either the GDH or the GS/GOGAT pathway or of both pathways simultaneously has been described (Kumar & Nicolas, 1984; Brown 1976; Kavanagh & Cole, 1976). To test which of the two enzyme systems is expressed in *Hyphomicrobium* ZV620, cells were grown in the chemostat under both C-[$s_0(NO_3^-N)$ = 0·09 g l^{-1}] and N-limitation [$s_0(CH_3OH)$ = 1·30 g l^{-1}]. The results in Table 1 clearly show that irrespective of the nature of the growth-limiting substrate, *Hyphomicrobium* ZV620 assimilated nitrogen from NO_3^- via GS/GOGAT exclusively, because complete repression of $NADP^+$-dependent GDH occurred under these conditions. The fact that *Hyphomicrobium* ZV620 exhibits an N-limited phenotype suggests that the reduction of NO_3^- to NH_4^+ by the assimilatory NO_3^- and NO_2^- reductases is the bottle-neck in N-assimilation during growth with NO_3^-.

DISCUSSION

Growth and cell composition

Although transition from C- to N-limited growth conditions is commonly encountered, only two detailed reports concerning such a transition exist (Hueting & Tempest, 1979; Egli & Quayle, 1986). These indicate that the transition does not occur in a stepwise manner but as a gradual adaption to the changing growth environment. The results reported here are consistent with the above findings and indicate the general existence of a regime of dual (C- and N-) limitation in between distinct C-limited and distinct N-limited growth.

Egli & Quayle (1986) demonstrated that the range of double substrate limitation can be predicted from the cellular composition using a simple empirical equation:

$$c_0/n_0 = Y_{X/N}/Y_{X/V}$$

where c_0 and n_0 are the C- and N-concentrations, respectively, in the feed, and $Y_{X/C}$ and $Y_{X/N}$ are the respective growth yield coefficients based on carbon and nitrogen measured under either distinctly C-limited or distinctly N-limited conditions. Applying this equation the results reported here ($Y_{X/C} = 0{\cdot}99$, $Y_{X/N} = 7{\cdot}19$, for C-limited growth and $Y_{X/C} = 0{\cdot}80$, $Y_{X/N} = 10{\cdot}30$, for N-limited growth) indicate that the region of dual substrate limitation should be between $7{\cdot}2 \leq C/N \leq 12{\cdot}8$, which corresponds to the values observed.

During growth under distinctly C-limited conditions the cellular composition remained essentially constant irrespective of the C:N ratio in the feed. Dual substrate limitation was characterized by a build-up of PHB in the cells, but once distinctly N-limited conditions were reached cellular composition again became constant but markedly different from the composition under methanol limitation. The drop in protein concentration is accounted for by the dilution effect resulting from enhanced PHB content. The results indicated that PHB accounts for 78% of the increase in dry weight, but as recovery of PHB in the analytical techniques used is less than 100% it is probable that PHB actually accounts for the whole increase in dry weight that was observed.

Bacterial growth theory implies that the $\mu_{max.}$ value obtained in exponentially growing batch cultures corresponds to the critical dilution rate at which cells are washed-out in chemostat cultures. In the case of *Hyphomicrobium* ZV620 such correspondence did not occur. The $\mu_{max.}$ during exponential batch growth was $0{\cdot}10\ h^{-1}$, irrespective of the initial methanol concentration ($0{\cdot}8$ to $4\ g\ l^{-1}$), whereas the critical dilution rate in the chemostat was $0{\cdot}14\ h^{-1}$. This effect was not due to wall growth (Topiwala & Hamer, 1971). The reduced $\mu_{max.}$ values measured in batch cultures indicate that residual methanol influences the growth rate of hyphomicrobia even at very low concentrations.

Specific activities of NH‡-assimilating enzymes

Both physiological and genetical aspects of NH‡-assimilation and the regulation of the enzymes involved have been comprehensively reviewed (Dalton, 1979; Brown, 1980; Magasanik, 1982). The essential conclusions of these authors are that in those organisms possessing both NH‡-assimilation pathways, the synthesis and activity of GDH and GS are regulated via the intracellular concentrations of 2-oxoglutarate, glutamine and glutamate: high 2-oxoglutarate and low glutamine concentrations increase the activity of GS and low concentrations of glutamate increase the activity of GDH. On the assumption that the intracellular NH‡-concentration reflects the extracellular NH‡-concentration the results reported here for *Hyphomicrobium* ZV620 agree with this general pattern. In *Klebsiella pneumoniae* repression of $NADP^+$-dependent GDH activity has been reported to be complete in the presence of GS/GOGAT activity, whereas considerable $NADP^+$-dependent-GDH activity was observed in *Escherichia coli* under such conditions. The pattern of expression of the $NADP^+$-dependent GDH and GS/GOGAT phenotypes in *Hyphomicrobium* ZV620 is similar to the one reported for *K. pneumoniae*, which suggests that the regulation of these enzymes at the genetic level might be similar in the two organisms (for details see Magasanik, 1982).

The degree of adenylylation of GS was shown to be primarily influenced by the nature of the growth-limiting substrate and was essentially independent of the growth rate. This finding is in contrast to the data reported for cells of *E. coli* grown under C-limitation, in which the degree of adenylylation was reported to increase with decreasing growth rates (Senior, 1975). In *Hyphomicrobium* ZV620 some 10–20% of GS was present in the active deadenylylated form during C-limited growth whilst during N-limited growth about 40% of GS was deadenylylated. In addition to this the concentration of GS protein also increased with decreasing N-availability. In *E. coli* (Senior, 1975) and *K. pneumoniae* (Janssen & Magasanik, 1977) up to 90% of GS has been reported to be deadenylylated. In cell-free extracts of *Hyphomicrobium* ZV620 we were unable to detect such high proportions of active GS. However, the possibility of adenylylation of GS during the harvesting and cell-breakage procedure cannot be excluded.

In contrast to results reported for the methylotrophic yeast *Hansenula polymorpha* (Egli & Quayle, 1986), in which the specific activity of GDH increased with decreasing NH_4^+-availability, the specific activity of GDH in methanol-grown *Hyphomicrobium* ZV620 decreased with decreasing NH_4^+-concentrations in the culture medium. However, this difference is probably a reflection of the dual assimilation system for NH_4^+ present in this bacterium. It should be noted that both GS and GDH are regulated in a stepwise manner, whereas GOGAT regulation occurs more gradually. An unexpected feature in *Hyphomicrobium* is the derepression of the synthesis of GDH under N-limited growth conditions at low growth rates, since this bacterium possesses the GS/GOGAT NH_4^+-assimilation system which would be expected to predominate under such conditions.

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