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SHORT COMMUNICATIONS

Glutamine Synthetase and Glutamate Synthase Activities During Growth and Sporulation in *Bacillus subtilis*

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Glutamine synthetase in *Bacillus subtilis* 168 was repressed to a greater extent by L-glutamine or L-arginine than by ammonia when each was used as sole nitrogen source. It was derepressed when either L-glutamate or nitrate was used as nitrogen source. Glutamate synthase was repressed by L-glutamate or L-arginine and, to a lesser extent, by L-glutamine but was derepressed during growth with ammonia or nitrate. Glutamine synthetase activity was unaltered during the onset of sporulation. Glutamate synthase activity, however, underwent a small and apparently transient increase in bacteria induced to sporulate by nitrogen limitation.

INTRODUCTION

The two main routes for the assimilation of ammonia in bacteria are the direct reductive amination of 2-oxoglutarate catalysed by glutamate dehydrogenase (GDH) and an energy-dependent coupled system involving glutamine synthetase (GS) and glutamate synthase (GOGAT) (Tempest *et al.*, 1970). In *Bacillus subtilis* 168, GDH activity is absent (Freese *et al.*, 1964; Fisher & Sonenshein, 1977; Pan, 1978) and ammonia is assimilated via the latter route (Elmerich, 1972). One aim of the present investigation was to examine the activity of the ammonia assimilating enzymes during growth of *B. subtilis* 168 under various conditions of nitrogen availability. Previous work with *B. subtilis* has only assessed the activity of these enzymes under a limited number of conditions (Freese *et al.*, 1964; Rebello & Strauss, 1969; Meers *et al.*, 1970; Deuel *et al.*, 1970) and none of the reports combined assays for both GS and GOGAT. Our second aim was to investigate the activities of GS and GOGAT during the onset of sporulation induced by either carbon or nitrogen deprivation. Sporulation in *Bacillus* species is usually triggered by starvation for a carbon or nitrogen source and ammonia limitation is a ready means of initiating the process (Schaeffer *et al.*, 1965; Dawes & Mandelstam, 1970). As ammonia is assimilated via the combined action of GS and GOGAT these enzymes might be responsible in some way for the repression of sporulation normally exerted by ammonia. The isolation of mutant strains of *B. subtilis* and *B. megaterium* which are both defective in GS activity and unable to sporulate has suggested that this enzyme might play a regulatory role in the initiation of sporulation (Reysset & Aubert, 1975; Bott *et al.*, 1977). In these studies and other reports of investigations of GS-defective strains of *B. subtilis* (Dean *et al.*, 1977; Fisher & Sonenshein, 1977), no clear distinction was generally made between carbon and nitrogen limitation as a means of promoting sporulation. As GS and GOGAT would be expected to play a role in sporulation via the metabolism of nitrogenous compounds, we have compared the activities of these enzymes during the onset of sporulation induced by either carbon or nitrogen starvation.

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METHODS

Organism and growth. Bacillus subtilis 168 (*trpC2*), which grows and sporulates normally in media containing L-tryptophan, was grown with aeration at 37 °C in minimal salts medium M34 (Schaeffer *et al.*, 1965) containing either 0·05% (w/v) NH_4Cl or alternative sources of nitrogen at 0·1% (w/v). Media were supplemented with 0·5% (w/v) D-glucose and 0·1% (w/v) trisodium citrate as carbon sources. Each medium is described by the nature of the nitrogen source, for example M34 (ammonia).

Conditions for obtaining sporulation. The procedure used was the resuspension method described by Sterlini & Mandelstam (1969). Bacteria were grown at 37 °C in a rich casein hydrolysate (CH) medium and sporulation was induced by resuspending them in an equal volume of minimal salts medium. To initiate sporulation by carbon deprivation, the original replacement medium of Sterlini & Mandelstam (1969) was used. This S(C) medium contained an adequate nitrogen source [0·05% (w/v) NH_4Cl and 0·01% (w/v) NH_4NO_3] but only a poor carbon source [0·15% (w/v) L-glutamate]. To initiate sporulation by nitrogen deprivation, a modified medium, S(N), was used; this was similar to the S(C) medium except that the NH_4Cl and NH_4NO_3 were replaced by 0·06% (w/v) $NaNO_3$ and L-glutamate was replaced by 0·2% (w/v) D-glucose. This medium therefore contained a good carbon source but only a poor nitrogen source. Resuspension of bacteria in either medium produced between 80 and 90% refractile spores in 7 to 8 h.

Measurement of growth and spore incidence. Growth was determined turbidimetrically by measuring A_{600} values with a Unicam SP500 spectrophotometer. Refractile spores were counted in the phase-contrast microscope.

Preparation of cell-free extracts. Samples (50 ml), harvested during the mid-exponential phase of growth (A_{600} 0·6 to 0·8) or at intervals after resuspension to initiate sporulation, were centrifuged at 4500*g* for 15 min at 4 °C and the pellet was taken up in 2·0 ml 0·05 M-Tris/HCl buffer (pH 7·4) containing 0·01 M-2-mercaptoethanol and 0·1 M-EDTA. This buffer had previously been shown to stabilize the GS of *B. subtilis* (Rebello & Strauss, 1969; Deuel *et al.*, 1970). Bacteria were disrupted using an MSE ultrasonic disintegrator for four 30 s periods with intervening cooling. Debris was removed by centrifugation at 75000*g* for 30 min at 4 °C and the supernatant was used as the source of enzyme activity.

Enzyme assays. Glutamate dehydrogenase [GDH; L-glutamate:$NADP^+$ oxidoreductase (deaminating); EC 1.4.1.4] and glutamate synthase [GOGAT; L-glutamate:$NADP^+$ oxidoreductase (transaminating); EC 1.4.1.13] were assayed spectrophotometrically at 30 °C by measuring NADPH oxidation at 340 nm according to Meers *et al.* (1970). The only modification was that 0·5 M-imidazole/HCl buffer (pH 7·5) was used and the controls were performed as recommended by Brenchley (1973). Endogenous NADPH oxidation was monitored with a reaction mixture lacking substrate, either L-glutamine or NH_4Cl. Specific activities, corrected for endogenous activity, are expressed as nmol NADPH oxidized min^{-1} (mg protein)$^{-1}$.

Glutamine synthetase [GS; L-glutamate:ammonia ligase (ADP-forming); EC 6.3.1.2] was assayed by measuring the formation at 30 °C of γ-glutamyl hydroxamate (Elliot, 1955). This assay measures the forward reaction of the enzyme to form L-glutamine from L-glutamate and ammonia in the presence of ATP and the enzyme forms γ-glutamyl hydroxamate when ammonia is replaced by hydroxylamine. Specific activities are expressed as nmol γ-glutamyl hydroxamate formed min^{-1} (mg protein)$^{-1}$ calibrated against standard solutions of γ-glutamyl hydroxamate (Sigma).

The protein content of cell-free extracts was determined by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Enzyme activities during growth

Bacillus subtilis grew at different rates in the presence of different nitrogen sources (Table 1). For a particular medium, the generation times varied slightly from one experiment to another, but the relative differences in growth rate promoted by the various nitrogen sources were reproducible. Cultures grew most rapidly with L-glutamine as nitrogen source, followed closely by ammonia and L-arginine. This suggested that L-glutamine, rather than ammonia, was the preferred nitrogen source, in agreement with the work of Baumberg & Harwood (1979). Nitrate, L-histidine and L-glutamate promoted much slower growth rates. Growth on nitrate may be limited by the rate at which ammonia is produced from it, as the intracellular pool of ammonia was low in *B. licheniformis* during growth with nitrate (Donohue & Bernlohr, 1978). The marked difference between L-arginine and L-histidine with regard to growth rate may be explained by the fact that D-glucose exerts a strong cata-

Table 1. *Effect of nitrogen source on the growth rate of B. subtilis and the activities of glutamine synthetase and glutamate synthase*

Extracts were prepared from bacteria harvested in the mid-exponential growth phase. The enzyme activities are an average of the total number of determinations shown, all of which were made using separate cultures. The values in parentheses indicate the range of activities found.

Nitrogen source in M34 medium	Doubling time (min)	Specific activity		No. of determinations
		Glutamine synthetase	Glutamate synthase	
Ammonia	50	170 (100–250)	740 (650–800)	12
Ammonia + L-arginine	54	63 (58–73)	130 (110–140)	3
Ammonia + L-glutamine	56	70 (60–88)	300 (180–400)	3
Ammonia + L-glutamate	60	116 (88–150)	340 (300–430)	3
Ammonia + L-histidine	60	138	700	1
Nitrate	144	516 (413–575)	400 (260–500)	3
L-Arginine	51	146 (88–150)	113 (100–130)	4
L-Glutamine	48	105 (63–130)	343 (250–500)	4
L-Glutamate	95	325	80	1
L-Histidine	360	ND	ND	—

ND, Not done.

bolite repression on the synthesis of the inducible L-histidine-degrading enzymes but not on the inducible enzymes degrading L-arginine (Chasin & Magasanik, 1968; Baumberg & Harwood, 1979).

No GDH activity, using NADPH or NADH as co-factor, was detected in bacteria grown in M34 (ammonia). This enzyme was not investigated further as it has a poor affinity for ammonia and would be expected to be most active during growth in this medium (Tempest *et al.*, 1970). The coupled activity of GS and GOGAT seems to be solely responsible for ammonia assimilation in *B. subtilis* because mutant strains devoid of either activity are unable to grow with ammonia as nitrogen source (Bott *et al.*, 1977; Fisher & Sonenshein, 1977; Pan, 1978).

Preliminary experiments with bacteria grown in M34 (ammonia) showed that more than 95% of the GS and GOGAT activities were in the soluble fraction. GS activity in the extract was stable at −20 °C for 24 h, but there was a 30% loss of GOGAT activity in this time. For this reason all assays were performed on freshly prepared extracts. GS activity had an absolute requirement for ATP. In the GOGAT assay, the activity with NADH was only 7% of that with NADPH. Obvious differences in GS and GOGAT activities were found in extracts from bacteria grown with different nitrogen sources (Table 1). However, there was some variation in the activity of both enzymes from separate cultures grown in the same medium and for this reason average values for each growth condition are presented together with the extreme activities found for each enzyme: small differences between average activities are therefore considered to be insignificant.

Synthesis of GS was derepressed during growth with a poor nitrogen source such as nitrate or L-glutamate. Conversely, GS activity was repressed when ammonia was used as nitrogen source and was further repressed when L-glutamine replaced ammonia or was used together with ammonia. These data, taken together with the observation that L-glutamine rather than ammonia is apparently the preferred nitrogen source, suggest that L-glutamine acts to repress GS synthesis. Thus, the high activity of GS found with nitrate or L-glutamate would presumably result from the low intracellular concentration of ammonia (Donohue & Bernlohr, 1978) which would limit the production of L-glutamine and, in turn, derepress GS synthesis. Repression of GS synthesis by L-arginine is less easy to explain. The pathway for L-arginine degradation in the conditions used here (a good carbon source and adequate aeration) is via L-ornithine to L-glutamate with the release of urea rather than ammonia

(De Hauwer *et al.*, 1964). As *B. subtilis* 168 can grow slowly with urea as sole nitrogen source (Harwood, 1974), we assume that sufficient ammonia can be produced when *B. subtilis* is growing rapidly with L-arginine to allow adequate formation of L-glutamine for the repression of GS synthesis.

Synthesis of GOGAT was derepressed when ammonia and, to a lesser extent, nitrate served as sole sources of nitrogen. It was most markedly repressed when either L-glutamate or L-arginine served as sole nitrogen source or were included together with ammonia. L-Glutamine appeared to be less effective than L-glutamate or L-arginine in repressing GOGAT synthesis, and L-histidine, when included with ammonia, had no obvious effect. These data suggest that GOGAT synthesis is repressed by L-glutamate as the metabolism of L-glutamine, L-arginine and L-histidine would be expected to occur via L-glutamate. The negligible effect of L-histidine was presumably due to the lack of induction of the necessary degradative enzymes in the presence of D-glucose.

Enzyme activities during the onset of sporulation

Sporulation may be initiated by different mechanisms under carbon or nitrogen limitation. In some Gram-negative bacteria, synthesis of certain inducible enzymes is promoted by cyclic AMP in combination with the cyclic AMP binding protein under conditions of carbon limitation and by GS under conditions of nitrogen limitation (Magasanik, 1977).

Sporulation was triggered by starvation for a carbon source in the S(C) medium and by starvation for a nitrogen source in the S(N) medium. When bacteria were transferred to these media from the CH medium, the A_{600} doubled (from about 0·5 to 1·0) in the first 5 h and then remained constant. Inclusion of D-glucose (0·2%, w/v) as an extra carbon source in the S(C) medium or L-glutamate (0·15%, w/v) as an extra nitrogen source in the S(N) medium promoted a rapid increase in A_{600} to a value well above that normally expected ($> 2{\cdot}0$). Presumably, addition of these supplementary carbon or nitrogen sources allowed the bacteria to resume vegetative growth rather than initiate sporulation. This was supported by the observation that no refractile spores were detected 5 h after resuspension when these additions were made to the normal S(C) or S(N) media, whereas 10 to 20% of cells normally showed refractile spores by this time in the unsupplemented media.

Specific activities of GS and GOGAT were determined in cell-free extracts prepared at intervals during the initial 2 h of resuspension in both S(C) and S(N) media. Samples were taken immediately upon resuspension and at 20 min intervals thereafter. Some variation in activity of both enzymes from separate experiments was again observed, but throughout each experiment only very slight differences in GS activity were found. These differences were not considered significant because of the variation in enzyme activity from one experiment to another. A small and transient increase in GOGAT activity was reproducibly found between 20 and 60 min after resuspension in either medium but was more pronounced in the S(N) medium [average specific activity of 48 nmol min^{-1} (mg protein)$^{-1}$ at 0 min rising to 112 at 30 min and falling to 45 by 120 min]. Limitation of growth rate in M34 (nitrate) led to a clear derepression of GS and GOGAT synthesis (Table 1). It was perhaps surprising to find a slight and apparently only transient increase in GOGAT activity in the S(N) medium which resembled M34 (nitrate) in having D-glucose as carbon source and nitrate as nitrogen source. The reason for the transient alteration in GOGAT activity and the lack of response of GS to nitrogen limitation under these sporulation conditions is not clear. Delayed induction or repression of the arginine catabolic and biosynthetic enzymes has been reported in *B. subtilis* (Vogel & Vogel, 1965; Baumberg & Harwood, 1979).

It is concluded that alteration in the rate of synthesis of GS plays no role in the initiation of sporulation either by carbon or nitrogen limitation. This conclusion is probably also valid for GOGAT because, although an increased activity of this enzyme was detected shortly after initiation, the increase was marginal in comparison with the 10-fold alteration in enzyme activity observed during growth. Using a different experimental approach, a

similar lack of correlation between GS specific activity and percentage sporulation has been reported in *B. licheniformis* (Donohue & Bernlohr, 1978).

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