Glutamine Synthetase Activities of Cultures of *Pseudomonas aeruginosa* Grown in Minimal Media with Histidine, Nitrate or Ammonium Sulphate as the Nitrogen Source

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INTRODUCTION

Potts & Clarke (1976) observed that when *Pseudomonas aeruginosa* strain PAC1 was grown in minimal medium with pyruvate as carbon source and histidine as nitrogen source, there was no catabolite repression of histidase when succinate was added to the medium unless ammonium salts were added at the same time. This strain has a defective histidine uptake system and growth is nitrogen limited in minimal medium with histidine as the sole nitrogen source. Strain PAC1 is unable to grow on histidine alone since histidine uptake is too slow to provide carbon and energy for growth although it is able to synthesize all the enzymes required for histidine catabolism specified by the *hut* genes. Glucose repression of histidase in *Klebsiella aerogenes* does not occur in glucose/histidine medium and this has been related to the increased activity of glutamine synthetase in this medium (Prival & Magasanik, 1971; Prival, Brenchley & Magasanik, 1973). Glutamine synthetase activates the transcription of the *hut* operon *in vitro* (Tyler, Deleo & Magasanik, 1974). The amount of glutamine synthetase is dependent on the nitrogen source in many bacteria and this enzyme may be essential for nitrogen assimilation when the available nitrogen, either as ammonium ions or as organic nitrogen, is present at very low concentrations (Meers, Tempest & Brown, 1970; Magasanik *et al.*, 1974). The histidine transport lesion of strain PAC1 provides a simple means of obtaining severe nitrogen limitation in a *P. aeruginosa* culture. We have investigated the glutamine synthetase activities of cultures grown in minimal medium, with the normal concentration of ammonium salts, and compared them with those of cultures grown under nitrogen limitation in histidine or nitrate medium. We have also examined cultures grown in a chemostat with growth limited by the availability of ammonium ions as the nitrogen source.

METHODS

Pseudomonas aeruginosa PAC1 (Kelly & Clarke, 1962; strain 8602/A) was maintained on nutrient agar. Strain PAC1R is a mutant resistant to lysis by bacteriophage Ps1 which occasionally causes lysis in slow-growing cultures. Minimal media were made up as described by Brammar & Clarke (1964), with the carbon and nitrogen sources indicated in Table 1. Batch cultures for enzyme assays were grown in a Biotech fermenter, capacity 300 ml, until the bacterial cell density reached an A_{670} of about 0.6. The inoculum consisted of 5 to 10 ml of a stationary culture in the same medium. The chemostat, constructed in this department, comprised a glass vessel with an integral water jacket of 100 ml capacity. The medium was supplied to the vessel via a Varioperpex pump (LKB); it was agitated in the course of aeration with a small aquarium pump and also by the action of a small magnetic 'flea'. Whenever the flow rate was changed, the culture was left to stabilize for at least 24 h. Samples of about 20 ml were taken for enzyme assays from the culture overflow, so that the time taken to collect them varied with the dilution rate. The sample bottle was kept on ice during the collection period. The cell density (A_{670}) over the range of growth rates examined was 0.33 to 0.35.

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Cultures were harvested by centrifugation and resuspended in 0.1 M-Tris/HCl buffer, pH 7.2. The bacteria were disrupted in a French pressure cell (Aminco) at 110 MPa and the debris was removed by centrifugation.

Protein was determined by the method of Lowry *et al.* (1951). Glutamine synthetase activity was measured by the method of Shapiro & Stadtman (1970). The exact details for this assay are important in assessing the significance of the results (Bender *et al.*, 1977). The procedure adopted in our experiments was as follows: The assay mixture was made up to 25 ml with 1.0 M-imidazole hydrochloride, 2.0 ml; 0.01 M-MnCl₂, 1.5 ml; 0.01 M-ADP, 1.5 ml; 0.067 M-glutamine, 15.0 ml; 1.0 M-sodium arsenate, 1.0 ml; 1.0 M-hydroxylamine hydrochloride, 1.0 ml; 1.0 M-NaOH, 1.0 ml; and distilled water [2.0 M-MgCl₂, 1.5 ml, was included for some assays]. The assay tubes contained 0.5 ml assay mixture and 0.1 ml (or more) enzyme preparation in 1.0 ml. Enzyme activity was linear for at least 30 min. The standard assay was carried out with incubation at 37 °C for 10 min. The reaction was stopped by addition of the ferric chloride reagent described by Shapiro & Stadtman (1970) and then A_{540} was measured. Activities are expressed as $\mu\text{mol } \gamma\text{-glutamylhydroxamate formed min}^{-1} (\text{mg protein})^{-1}$ in the cell extract. Glutamate synthase and glutamate dehydrogenase activities were measured by the methods of Cole *et al.* (1974).

RESULTS AND DISCUSSION

Growth of *P. aeruginosa* PAC1 in succinate/histidine or pyruvate/histidine medium was slow and the cultures became very sensitive to additional environmental stress. Some experiments were abandoned because of cell lysis which occurred more frequently when the cultures had been exposed to fluorescent light for long periods. It was found most convenient to arrange for the major period of growth to occur overnight so that the cultures could be harvested during the following morning as soon as they had reached the required cell density. With succinate/histidine and pyruvate/histidine media this took about 20 h (± 2 h). For some experiments strain PAC1R was used; this had a slightly faster growth rate in these media, reaching the required cell density in about 16 h or less. (This may mean that the resistance to bacteriophage Ps1 is associated with a surface change which also has the effect of allowing it to take up histidine slightly better than the parent strain.) The cultures grown with nitrate took about 10 to 12 h to reach a cell density equivalent of 0.6. The cultures growing in the NH₄⁺ media took 5 to 7 h and were in the exponential growth phase, whereas growth of the histidine and nitrate cultures was almost linear.

Cultures grown in the fermenter in minimal media with histidine or nitrate as nitrogen source had higher glutamine synthetase activities than those grown with 10 mM-(NH₄)₂SO₄ (Table 1). This had been expected and was consistent with the presumed role of glutamine synthetase in the relief of succinate repression of histidase synthesis in nitrogen-limited medium. [We were concerned about the variation between separate experiments under the same growth conditions, a problem which has been encountered by other workers studying glutamine synthetase in the enterobacteria (Brenchley, Baker & Patil, 1975; Bender *et al.*, 1977). We have therefore given the results of single experiments rather than the mean of a series. All assays were carried out in duplicate and the results were reproducible for each preparation within, at most, $\pm 5\%$.] Also included in Table 1 are the results from experiments with strain PAC1R grown in the fermenter and PAC1 grown in conical flasks on a shaker. The flask cultures of PAC1 growing in succinate/histidine medium were less susceptible to spontaneous lysis than those growing in the fermenter.

The total glutamine synthetase activities of PAC1 cultures grown in NH₄⁺ media, and for PAC1 grown in the succinate/histidine medium in a shaken flask, are within the range reported for enterobacteria grown in nitrogen-excess and nitrogen-limited media: Bloom, Streicher & Tyler (1977) reported values of 0.22 and 1.29, respectively, for *Klebsiella aerogenes* and 0.10 and 1.45 for *Salmonella typhimurium*. These results, with about 10-fold differences, are similar to those reported by other workers using a variety of methods to obtain nitrogen-limited growth. We had previously found little difference in total glutamine synthetase activities of flask cultures of strains PAC1 or PAC1R grown with concentrations of (NH₄)₂SO₄ ranging from 1 to 100 mM but invariably found activities about 10-fold higher in cultures grown in succinate/histidine medium.

Table 1. *The effect of the nitrogen source on glutamine synthetase activities of Pseudomonas aeruginosa*

Cultures were grown in minimal medium with the carbon (1 %, w/v) and nitrogen (10 mM) sources indicated. Glutamine synthetase activities were measured in cell extracts and are expressed as $\mu\text{mol } \gamma\text{-glutamylhydroxamate formed min}^{-1} (\text{mg protein})^{-1}$.

Strain	Nitrogen source	Carbon source	Glutamine synthetase activities					
			Expt no.					
<i>Batch cultures</i>			1	2	3	4		
PAC1	NH ₄ ⁺	Succinate	0.16	0.36	0.24			
PAC1	NH ₄ ⁺	Pyruvate	0.96	0.19				
PAC1	Histidine	Succinate	4.4	4.5	3.6	5.0		
PAC1	Histidine	Pyruvate	2.1	1.8				
PAC1	Nitrate	Succinate	4.5	5.6	4.2			
PAC1	NH ₄ ⁺ /Nitrate	Succinate	0.8	0.3				
PAC1R	NH ₄ ⁺	Succinate	0.18					
PAC1R	Histidine	Succinate	1.82					
PAC1 (flask)	NH ₄ ⁺	Succinate	0.10					
PAC1 (flask)	Histidine	Succinate	1.70					
			Dilution rate (h ⁻¹)					
<i>Chemostat culture (nitrogen limited)</i>			0.01	0.22	0.30	0.36	0.51	0.68
PAC1R	NH ₄ ⁺	Succinate	0.56	0.79	0.69	0.57	0.33	0.32

Glutamine synthetase activities of PAC1 grown in succinate/histidine medium in the fermenter were significantly higher than those obtained in flask culture. The nitrogen limitation of the PAC1 cultures in the succinate/histidine medium was severe, as evidenced by the low growth rate, and under these conditions the glutamine synthetase may be completely derepressed. The activities of the succinate/histidine cultures were about twice those of the pyruvate/histidine cultures. In minimal salt media with 0.1 % (NH₄)₂SO₄, succinate can support a doubling time of about 50 min at 37 °C and pyruvate about 90 min. This may mean that the internal pool of available nitrogen in the succinate/histidine cultures is lower than that in the pyruvate/histidine cultures and allows greater derepression of glutamine synthetase.

The chemostat cultures were limited by the NH₄⁺ supply, but this did not lead to particularly elevated levels of glutamine synthetase, a result which was consistent with the inability to obtain elevated levels of glutamine synthetase in batch culture with low concentrations of (NH₄)₂SO₄. However, there were significant variations over the growth rates studied with a maximum at a dilution rate of 0.25 h⁻¹. Variations have also been observed in glutamine synthetase activities of *Pseudomonas putida* in chemostat culture over a range of dilution rates with growth limited by the NH₄⁺ concentration (P. D. Laverack, personal communication).

Glutamine synthetase is regulated in activity by reversible adenylylation (Stadtman *et al.*, 1970). The assay developed for measuring the state of adenylylation of the *Escherichia coli* enzyme depended on the observation that the standard assay in the presence of Mn²⁺ measured the total activity and the assay in the presence of Mn²⁺ and Mg²⁺ measured only the biosynthetically active, non-adenylylated form. The assay procedure was complicated by the finding that activities of the non-adenylylated and adenylylated species varied with pH but not to the same extent. It was also observed that the non-adenylylated form might be activated by Mg²⁺. Recently, Bender *et al.* (1977) have re-examined the glutamine synthetase of *K. aerogenes* and point out that it differs from that of *E. coli* in several respects. An important finding was that the pH isoactivity point of the two forms of the *K. aerogenes* enzyme was quite different from that of the *E. coli* enzyme and that it was altered by

changes in the components of the reaction mixture. They also observed that the apparent glutamine synthetase activities changed during harvesting of the cultures. Previously, Brenchley *et al.* (1975) had reported that the glutamine synthetase from cells of *K. aerogenes* and *S. typhimurium* harvested in exponential growth phase was non-adenylylated, irrespective of whether they were grown with nitrogen-excess or nitrogen-limitation. These workers found little or no difference in assays in the presence and absence of Mg^{2+} with extracts from the exponentially growing cells, although with the stationary phase cells there were marked differences in the presumed state of adenylylation.

Tronick, Ciardi & Stadtman (1973) reported that *P. aeruginosa* grown in minimal media with high and low concentrations of NH_4^+ showed differences in the adenylylation state of glutamine synthetase, and we hoped that these differences would be apparent in our experiments. All the assays listed in Table 1 were also carried out in the presence of Mg^{2+} but we have not included the values obtained since we are uncertain of the significance that should be attached to them. There were only slight differences in glutamine synthetase activities measured in the presence or absence of Mg^{2+} over the pH range 6.8 to 7.5 so that although an isoactivity point had not been established it seemed reasonable to make comparisons using the standard assay conditions at pH 7.1. All the extracts from the fermenter cultures grown in histidine-limiting medium had much lower activities (about a third) in the presence of Mg^{2+} and this could mean that most of the glutamine synthetase was adenylylated. The glutamine synthetase activities of the cultures grown with NH_4^+ were also somewhat lower in the presence of Mg^{2+} in most cases. On the other hand, our previous series of experiments in which strain PAC1 had been grown in histidine medium in shaken flasks had shown that the glutamine synthetase activities of the nitrogen-limited cultures were higher in the presence of Mg^{2+} , i.e. the non-adenylylated activity was greater than the 'total' activity. Bender *et al.* (1977) have shown that at some pH values the non-adenylylated form of glutamine synthetase of *K. aerogenes* is markedly activated by Mg^{2+} and we suspect that this is also true for the enzyme from *P. aeruginosa*. Activation by Mg^{2+} was most apparent in our experiments with the extracts prepared from chemostat cultures where the glutamine synthetase activities doubled in the presence of Mg^{2+} . Newman & Cole (1977) observed Mg^{2+} stimulation of glutamine synthetase in nitrogen-limited chemostat cultures of *E. coli*.

The discrepancies in the Mg^{2+} effects on glutamine synthetase activities of *P. aeruginosa* grown under various conditions in nitrogen-excess and nitrogen-limited media are similar to those discussed by Bender *et al.* (1977) for *K. aerogenes* and we feel unable to reach conclusions on the adenylylation states of the *P. aeruginosa* enzyme without further study. The main problems appear to be (i) the adenylylation state may change during harvesting and the extent to which this happens may depend on the medium on which the culture has been grown, (ii) Mg^{2+} may activate one form and inhibit the other and (iii) there may be differences in the pH isoactivity points and these may vary with the other components of the assay mixture.

In some of the experiments reported in Table 1 the activities of glutamate dehydrogenase and glutamate synthase were also measured. The variations in glutamate synthase were not significant but the glutamate dehydrogenase activities of the succinate/ NH_4^+ culture were 10-fold higher than those for the succinate/histidine and succinate/nitrate cultures [0.70, 0.05 and 0.07 $\mu\text{mol NADH oxidized min}^{-1} (\text{mg protein})^{-1}$, respectively].

We conclude that the total amount of glutamine synthetase produced by *P. aeruginosa* varies inversely with the available nitrogen and that it is probably important in nitrogen assimilation. The correlation of high glutamine synthetase activities and relief from catabolite repression in minimal media with histidine as the growth-limiting nitrogen source suggests that glutamine synthetase plays an active role in the regulation of the *hut* genes of *P. aeruginosa*.

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