The Enzymes of Ammonia Assimilation and their Control in Members of the Genus *Erwinia*

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The genus *Erwinia* is heterogeneous with respect to the presence of the ammonia assimilating enzymes glutamine synthetase (GS), NADP-linked glutamate synthase (GOGAT) and NADP-linked glutamate dehydrogenase. Three major groups were distinguished; one showed activity of all three enzymes, the second only GS and GOGAT activity and the third only GS activity. Aspartase did not appear to be significant in ammonia assimilation in any group.

INTRODUCTION

Nitrogen is essential for the growth of micro-organisms and, therefore, an understanding of its assimilation, especially from inorganic sources, is of fundamental importance. Whatever the primary source of nitrogen, ammonia plays a central role in this assimilation. Many pathways for the incorporation of ammonia into organic acceptors have been implicated in bacteria, but two routes are of major importance (Brown, Macdonald-Brown & Meers, 1974; Brown & Johnson, 1977). These are the long recognized NADP-linked glutamate dehydrogenase (GDH) and the more recently discovered, energy-dependent coupled system of glutamine synthetase (GS) and NADP- or NAD-linked glutamate synthase (GOGAT). Both pathways result in the net synthesis of one molecule of glutamate from ammonia. The significance of the GS/GOGAT couple lies in its greater efficiency at low ammonia concentrations due to the high affinity of GS for ammonia.

The phytopathogenic genus *Erwinia*, in its latest classification (Lelliott, 1974), consists of 15 species, grouped into the 'amylovora', 'carotovora' and 'herbicola' clusters; this subdivision is largely based upon acid production in peptone water sugars and a further series of biochemical tests. Phytopathogenic bacteria have, in general, been neglected from a physiological point of view and little data is available concerning ammonia assimilation in *Erwinia* species. In an organism the identity of which is in doubt, but which is likely to be *E. carotovora* var. *carotovora*, GDH was detected although it was suggested that aspartase was probably responsible for the assimilation of ammonia (Grula *et al.*, 1968). In a strain (MRE 604) of *E. carotovora* examined by Meers, Tempest & Brown (1970), NADP-GOGAT activity was present but GDH was not detected.

It was in view of the paucity of data concerning ammonia assimilation in this potentially economically important genus that the present study was undertaken. This communication reports upon a comparative systematic study of the mechanisms of ammonia assimilation, and their control, in representative members of the genus *Erwinia* grown in batch cultures and in the more physiologically demanding continuous (chemostat) cultures.

METHODS

Bacterial strains. The bacteria used in this study were obtained from the National Collection of Plant Pathogenic Bacteria (NCPBB), Harpenden, Hertfordshire, and were as follows (NCPBB numbers given in parentheses). 'Amylovora' cluster: *E. amylovora* (595), *E. salicis* (447), *E. tracheiphila* (2452), *E. nigrifluens* (564), *E. quercina* (1852), *E. rubrifaciens* (2020). 'Herbicola' cluster: *E. herbicola* var. *herbicola* (660), *E. herbicola* var. *ananas* (441), *E. stewartii* (449), *E. uredovora* (1416). 'Carotovora' cluster: *E. carotovora* var. *carotovora* (312), *E. carotovora* var. *atroseptica* (549), *E. chrysanthemi* (402), *E. cyripedii* (750), *E. rhapontici* (139).

They were maintained by fortnightly subculture on nutrient agar slopes [Lab lemco, 0.8 % (w/v); peptone, 1.0 % (w/v); NaCl, 0.5 % (w/v); Lab M agar, 2.0 % (w/v)] or yeast extract agar slopes [yeast extract, 0.7 % (w/v); peptone, 1.0 % (w/v); D-glucose, 1.0 % (w/v); NaCl, 0.5 % (w/v); Lab M agar, 2.0 % (w/v)].

Growth conditions. Defined liquid growth media were adapted from those described by Starr (1946*a, b*; 1949) and Starr & Mandel (1950).

Batch cultures. The medium contained (mmol l⁻¹ in distilled water): D-glucose, 14; MgSO₄·7H₂O, 0.8; KH₂PO₄, 15; NaCl, 8.6; NH₄Cl or KNO₃ or Casamino acids (as glutamic acid equivalent), 3.6. It was autoclaved at 115 °C for 20 min (D-glucose and KH₂PO₄ were autoclaved separately), and then sterile solutions of trace elements and vitamins were added aseptically to give the following final concentrations (μmol l⁻¹): H₃BO₃, 0.081; KI, 0.006; CuSO₄·5H₂O, 0.036; FeSO₄·7H₂O, 1.3; MnSO₄·H₂O, 0.09; MoO₃, 0.069; ZnSO₄·7H₂O, 0.17; CaCO₃, 100; thiamin hydrochloride, 0.0089; biotin, 0.0045; nicotinic acid, 1.6; calcium pantothenate, 0.42; riboflavin, 0.13; cyanocobalamin, 0.003; *p*-aminobenzoic acid, 0.37; and folic acid, 0.23. The final pH of the medium was 6.8. Cultures were grown in 1 litre medium in 2 litre Erlenmeyer flasks in an orbital shaker incubator (Gallenkamp) at 30 °C and 150 rev. min⁻¹.

Continuous cultures. The media contained (mmol l⁻¹ in distilled water): for ammonia-limited conditions, D-glucose, 67; MgSO₄·7H₂O, 4.1; NaCl, 8.6; KH₂PO₄, 15; NH₄Cl, 7.2; and for ammonia-excess (glucose-limited) conditions, D-glucose, 14; MgSO₄·7H₂O, 4.1; NaCl, 8.6; KH₂PO₄, 15; NH₄Cl, 36. The media were autoclaved (D-glucose and KH₂PO₄ were autoclaved separately) and trace elements and vitamins were added as described above. Cultures were grown in a Porton-type chemostat of working volume 1 litre at 30 °C and at a dilution (= growth) rate of 0.05 h⁻¹. pH was maintained at 6.8 by the automatic addition of sterile 2 M-sodium hydroxide, and the vessel was sparged with sterile air at 1 litre min⁻¹.

Preparation of cell-free (enzyme) extracts. Batch cultures were harvested in the late-exponential phase of growth and chemostat cultures directly from the culture vessel. Samples were centrifuged at 4500 g for 15 min at 4 °C, and the pellet was washed once in 100 mM-phosphate buffer (pH 7.8), centrifuged as before, and finally resuspended in a small volume of phosphate buffer containing 1 mM-2-mercaptoethanol.

Bacteria were disrupted using either an ultrasonic disintegrator (MSE) for two 20 s periods, with intervening cooling, or an X-Press (Biotec, Stockholm, Sweden) for three cycles. Unbroken bacteria and debris were removed by centrifuging at 75000 g for 30 min at 4 °C. Low molecular weight metabolites were removed from the resulting extract by passage through a column (30 × 2 cm) of Sephadex G-50 at 4 °C. 2-Mercaptoethanol was added to the extract to give a final concentration of 1 mM.

Enzyme assays. Glutamate dehydrogenase [GDH; L-glutamate:NADP⁺ oxidoreductase (deaminating); EC 1.4.1.4] and glutamate synthase [GOGAT; L-glutamine:2-oxoglutarate aminotransferase (NADPH-oxidizing); EC 2.6.1.53] were assayed spectrophotometrically by measuring NAD(P)H oxidation at 340 nm. The assay system contained (in 3.0 ml): 200 μmol phosphate buffer (pH 7.8); 0.1 μmol NAD(P)H; 10 μmol 2-oxoglutarate; and enzyme preparation. The endogenous (non-specific) rate of oxidation was monitored using this mixture, and then either 100 μmol NH₄Cl or 30 μmol glutamine (freshly prepared solution) was added to start the reaction and the specific rate of oxidation was measured.

Enzymes analogous to GDH and GOGAT, but using different acceptor molecules, were also assayed by the above method except that one of the following was substituted for the 2-oxoglutarate: 10 μmol pyruvate; 10 μmol oxaloacetate; 10 μmol 2-oxobutyrate; 40 μmol 2-oxoisovalerate. Specific activities are expressed as nmol NAD(P)H 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 of γ-glutamyl hydroxamate at pH 7.8 (imidazole/HCl buffer), as described by Elliott (1955). Specific activities are expressed as nmol γ-glutamyl hydroxamate formed min⁻¹ (mg protein)⁻¹, calibrated against a standard solution of γ-glutamyl hydroxamate.

Aspartase [L-aspartate ammonia-lyase; EC 4.3.1.1] was assayed using a modification of the method described by Halpern & Umbarger (1960) and Marcus & Halpern (1969). The assay system contained (in 3.0 ml): 200 μmol phosphate buffer (pH 7.8); 1 μmol MgSO₄·7H₂O; 40 μmol potassium aspartate; and enzyme preparation. The ammonia liberated was estimated by the method of Fawcett & Scott (1960). Specific activities are expressed as nmol ammonia released min⁻¹ (mg protein)⁻¹.

Glutaminase [L-glutamine amidohydrolase; EC 3.5.1.2] was assayed in the same way as aspartase except that $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and potassium aspartate were replaced by $0.3 \mu\text{mol}$ EDTA and $30 \mu\text{mol}$ glutamine (freshly prepared solution), respectively.

Protein content. The protein contents of cell-free extracts were determined using the Folin-Ciocalteu reagent as described by Lowry *et al.* (1951).

RESULTS

Batch cultures studies

All members of the genus *Erwinia* studied grew in defined glucose/salts medium with ammonia as nitrogen source, showing maximum specific growth rates in the range 0.17 h^{-1} to 0.39 h^{-1} . All members of the 'carotovora' cluster utilized nitrate as sole nitrogen source whereas none of the 'amylovora' cluster (except *E. tracheiphila*) and only some of the 'herbicola' cluster could do so. Casamino acids or glutamate, provided as sole source of nitrogen, supported growth of all species.

The activities of GS, NADP-GOGAT and NADP-GDH in extracts of members of the 'herbicola', 'carotovora' and 'amylovora' clusters, grown with different sources of nitrogen, are shown in Table 1. NAD-GOGAT, NAD-GDH and corresponding amido-transferases and dehydrogenases (both NAD- and NADP-linked) able to utilize pyruvate, oxaloacetate, oxobutyrate, or oxoisovalerate were not detected in any cultures. Although heterogeneity with respect to the presence and activity of enzymes was apparent, the presence of GS was a common feature of all cultures examined. In general, GS activities were higher (up to 60 %) during growth on Casamino acids than on either nitrate or ammonia.

In members of the 'herbicola' cluster, GS was the only ammonia assimilating enzyme detected. In both the 'carotovora' and 'amylovora' clusters, however, a more complex pattern of enzyme distribution was seen. Both *E. carotovora* var. *carotovora* and *E. carotovora* var. *atroseptica* possessed GOGAT and GDH, the activities of both enzymes being higher during growth on ammonia than on the other nitrogen sources. In *E. chrysanthemi* only GOGAT was detected whilst in *E. rhapontici* only GDH was present (and only during growth on ammonia). *Erwinia cypripedii*, however, showed only GS activity and was thus similar to species in the 'herbicola' cluster. In the 'amylovora' cluster, the type species, *E. amylovora*, showed only GS activity; *E. salicis*, *E. nigrifluens*, *E. quercina* and *E. rubrifaciens* showed GOGAT activity but not GDH; and *E. tracheiphila* showed both GOGAT and GDH activities.

These findings, which demonstrated the heterogeneity of the genus *Erwinia* with respect to ammonia assimilating enzymes and, in some species, the apparent absence of a mechanism for the assimilation of ammonia, prompted a more physiologically demanding study employing continuous (chemostat) cultures.

Continuous culture studies

Cultures were established under two nitrogen nutrient regimes, namely, ammonia limitation and ammonia excess coupled with glucose limitation. It was believed that such an approach would elucidate not only which mechanisms of ammonia assimilation were operative but also the control of their expression. Of the enzymes which had been assayed in batch cultures, only those which had been detected (i.e. GS, NADP-GOGAT and NADP-GDH) were measured in extracts obtained from continuous cultures. In addition, aspartase and glutaminase were assayed, the former because it is a putative ammonia assimilating enzyme and the latter to eliminate the possibility that any observed NADP-GOGAT activity might be due to the combined effects of glutaminase and NADP-GDH. In no case could an observed GOGAT activity be ascribed to such combined activities.

All members of the 'herbicola' cluster showed GS activity (Table 2), with much higher activities under ammonia-limited conditions than under ammonia-excess conditions. GOGAT and GDH activities were not detected. Aspartase activity was present except in

Table 1. Activities of ammonia assimilating enzymes in batch-grown cultures of *Erwinia* spp.

Organism	Nitrogen source*	Specific activity†		
		GS	NADP-GOGAT	NADP-GDH
‘Herbicola’ cluster				
<i>E. herbicola</i>	Nitrate	NG	NG	NG
var. <i>herbicola</i>	Ammonia	5	ND	ND
	Casamino acids	17	ND	ND
<i>E. herbicola</i>	Nitrate	11	ND	ND
var. <i>ananas</i>	Ammonia	8	ND	ND
	Casamino acids	17	ND	ND
<i>E. stewartii</i>	Nitrate	NG	NG	NG
	Ammonia	2	ND	ND
	Casamino acids	28	ND	ND
<i>E. uredovora</i>	Nitrate	7	ND	ND
	Ammonia	11	ND	ND
	Casamino acids	7	ND	ND
‘Carotovora’ cluster				
<i>E. carotovora</i>	Nitrate	17	8	11
var. <i>carotovora</i>	Ammonia	10	17	448
	Casamino acids	37	ND	32
<i>E. carotovora</i>	Nitrate	21	17	9
var. <i>atroseptica</i>	Ammonia	6	26	108
	Casamino acids	11	15	ND
<i>E. chrysanthemi</i>	Nitrate	11	14	ND
	Ammonia	16	40	ND
	Casamino acids	7	5	ND
<i>E. cypripedii</i>	Nitrate	11	ND	ND
	Ammonia	4	ND	ND
	Casamino acids	17	ND	ND
<i>E. rhapontici</i>	Nitrate	9	ND	ND
	Ammonia	6	ND	24
	Casamino acids	17	ND	ND
‘Amylovora’ cluster				
<i>E. amylovora</i>	Nitrate	NG	NG	NG
	Ammonia	3	ND	ND
	Casamino acids	42	ND	ND
<i>E. salicis</i>	Nitrate	NG	NG	NG
	Ammonia	6	16	ND
	Casamino acids	21	4	ND
<i>E. tracheiphila</i>	Nitrate	14	9	36
	Ammonia	11	ND	172
	Casamino acids	11	42	13
<i>E. nigrifluens</i>	Nitrate	NG	NG	NG
	Ammonia	4	25	ND
	Casamino acids	15	5	ND
<i>E. quercina</i>	Nitrate	NG	NG	NG
	Ammonia	16	34	ND
	Casamino acids	37	ND	ND
<i>E. rubrifaciens</i>	Nitrate	NG	NG	NG
	Ammonia	7	10	ND
	Casamino acids	18	4	ND

NG, No growth; ND, not detected, i.e. < 1 nmol min⁻¹ (mg protein)⁻¹.

* For media, see Methods.

† See Methods.

Table 2. Influence of the concentration of ammonia on the activities of ammonia assimilating enzymes in chemostat cultures of *Erwinia* spp.

Organism	Growth condition*	Specific activity†			
		GS	NADP-GOGAT	NADP-GDH	Aspartase
‘Herbicola’ cluster					
<i>E. herbicola</i>	Ammonia-limited	34	ND	ND	ND
var. <i>herbicola</i>	Ammonia-excess	5	ND	ND	411
<i>E. herbicola</i>	Ammonia-limited	66	ND	ND	99
var. <i>ananas</i>	Ammonia-excess	7	ND	ND	71
<i>E. stewartii</i>	Ammonia-limited	93	ND	ND	50
	Ammonia-excess	5	ND	ND	446
<i>E. uredothora</i>	Ammonia-limited	61	ND	ND	ND
	Ammonia-excess	5	ND	ND	264
‘Carotovora’ cluster					
<i>E. carotovora</i>	Ammonia-limited	59	48	7	15
var. <i>carotovora</i>	Ammonia-excess	3	11	100	51
<i>E. carotovora</i>	Ammonia-limited	24	30	21	647
var. <i>atroseptica</i>	Ammonia-excess	4	9	69	510
<i>E. chrysanthemi</i>	Ammonia-limited	55	23	ND	ND
	Ammonia-excess	6	8	ND	139
<i>E. cypripedii</i>	Ammonia-limited	6	ND	ND	363
	Ammonia-excess	1	ND	ND	275
<i>E. rhapontici</i>	Ammonia-limited	49	ND	3	ND
	Ammonia-excess	5	ND	13	ND
‘Amylovora’ cluster					
<i>E. amylovora</i>	Ammonia-limited	92	ND	ND	12
	Ammonia-excess	11	ND	ND	110
<i>E. salicis</i>	Ammonia-limited	36	9	ND	ND
	Ammonia-excess	10	9	ND	74
<i>E. nigrifluens</i>	Ammonia-limited	92	41	ND	319
	Ammonia-excess	3	24	ND	105
<i>E. quercina</i>	Ammonia-limited	60	38	ND	ND
	Ammonia-excess	8	8	ND	ND
<i>E. rubrifaciens</i>	Ammonia-limited	46	15	ND	ND
	Ammonia-excess	11	6	ND	ND

ND, Not detected, i.e. < 1 nmol min⁻¹ (mg protein)⁻¹.

* For media, see Methods.

† See Methods.

ammonia-limited cultures of *E. herbicola* var. *herbicola* and *E. uredoovora* and was generally greater when ammonia was provided in excess.

GS activity was also present in all members of the 'carotovora' cluster (Table 2) and, again, activities were higher under conditions of ammonia-limited growth. As previously observed in batch-grown cultures with ammonia (Table 1), *E. carotovora* var. *carotovora* and var. *atroseptica* showed activities of both GOGAT and GDH. Reciprocal control of these enzymes appeared to operate in that high GOGAT/low GDH levels were obtained under ammonia-limited conditions whilst the reverse was true when ammonia was present in excess. In *E. chrysanthemi* only GOGAT activity was present and showed similar control to that seen in strains of *E. carotovora*, whilst in *E. rhapontici* GDH was present and at a higher level under conditions of ammonia excess. *Erwinia cypripedii*, however, showed neither GOGAT nor GDH activity thus confirming the batch culture results. Aspartase activity was detected in all organisms of this cluster except *E. rhapontici*, but no real pattern was apparent.

Members of the 'amylovora' cluster (Table 2) all showed GS activity with a similar pattern of control. Again, as observed in batch cultures (Table 1), *E. amylovora* showed no activity of either GOGAT or GDH whilst the other members of the group showed only GOGAT activity with higher levels being observed under conditions of ammonia limitation. Aspartase activity was only detected in *E. amylovora*, *E. salicis* and *E. nigrifluens*.

DISCUSSION

The present study has established that the genus *Erwinia* is heterogeneous with respect to the mechanisms of ammonia assimilation and, further, that such heterogeneity exists within the individual 'carotovora' and 'amylovora' clusters. The erwinias can be broadly divided into three groups with respect to the presence of the recognized ammonia assimilating enzymes GS, GOGAT and GDH.

The first group comprises organisms like *E. carotovora* which assimilate ammonia by either the GS/GOGAT or GDH routes depending upon the ammonia concentration in the medium. Reciprocal control of these pathways is operative in that under conditions of ammonia limitation GS and GOGAT activities are high and that of GDH is low, whilst under conditions of ammonia excess the reverse is true. Although this behaviour is typical of that previously reported for several bacteria, e.g. *Klebsiella (Aerobacter) aerogenes* (Meers *et al.*, 1970; Brenchley, Prival & Magasanik, 1973), it differs from that reported for *E. carotovora* MRE 604 in which GDH activity was not detected (Meers *et al.*, 1970). Such reciprocal control makes metabolic sense in an organism which may, in its natural environment, be exposed to fluctuating concentrations of ammonia.

The second group comprises organisms such as *E. salicis*, *E. nigrifluens*, *E. quercina* and *E. rubrifaciens* which apparently assimilate ammonia only by the GS/GOGAT route. These enzymes appear to be controlled in a fashion similar to that in *E. carotovora*, showing decreased activities under conditions of ammonia excess. In the absence of GDH, such behaviour raises questions as to the significance of the GS/GOGAT route under conditions of ammonia excess. The possession of this single high affinity pathway might indicate that, under natural conditions, these organisms normally only encounter low concentrations of ammonia and that under high concentrations of ammonia, efficiency of growth is reduced. Alternatively, some other undetected low affinity pathway may replace GDH when ammonia concentrations are high.

The third group, which includes *E. amylovora*, *E. cypripedii* and the 'herbicola' cluster, shows only GS activity. Since GS alone cannot account for the net assimilation of ammonia, an alternative route must operate. It has been suggested that aspartase may be responsible for ammonia assimilation in *Erwinia* sp. (Grula *et al.*, 1968), but, from studies on *Escherichia coli*, the biosynthetic role of aspartase is in doubt (Halpern & Umbarger, 1960; Vender & Rickenberg, 1964; Vender, Jayaraman & Rickenberg, 1965; Marcus & Halpern, 1969). This, taken with the view of Tosa *et al.* (1977) that the enzyme in *Escherichia coli* may be membrane-bound, and the present findings that (i) aspartase activities in GOGAT/GDH negative organisms are not substantially different from those in GOGAT/GDH positive organisms, and (ii) aspartase activities are generally decreased under ammonia-limited growth conditions, suggest that the primary function of aspartase is catabolic. Thus, in the third group of erwinias, a hitherto unrecognized pathway of ammonia assimilation may exist. Further studies are being undertaken to investigate this possibility.

The one enzyme consistently detected in all cultures was GS, which is perhaps not surprising in view of the multiple biosynthetic requirements for glutamine. The control of synthesis of this enzyme was similar in all species examined. The relatively high level of GS in cultures grown in the presence of amino acids agrees well with the suggestions that amino acids, provided as sole nitrogen source, produce conditions of nitrogen limitation (Mecke & Holzer, 1966; Woolfolk, Shapiro & Stadtman, 1966) and that glutamate can act

as an inducer of GS synthesis (Wu & Yuan, 1968). The consistently observed derepression of GS synthesis upon transfer from ammonia-excess to ammonia-limited conditions is in keeping with previous findings in many bacteria (Meers *et al.*, 1970; Shapiro & Stadtman, 1970; Brenchley *et al.*, 1973; Brown *et al.*, 1974).

In conclusion, the present study has shown a complex pattern with respect to ammonia assimilating enzymes in the genus *Erwinia*. Some members are like many other bacteria in that they possess GS and either GOGAT or GDH or both, whilst in other members there was no detectable pathway of ammonia assimilation. Findings such as these stress the need for more systematic approaches in studies of this type rather than restricting attention to a single 'representative' species.

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