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Growth of *Pseudomonas aeruginosa* Mutants Lacking Glutamate Synthase Activity

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Mutant strains SU1, SU4, and US1 lacking glutamate synthase (GOGAT) activity were isolated from strains of *P. aeruginosa* for which histidine is a growth rate-limiting source of nitrogen. Strains SU1 and SU4 were unable to grow when a low concentration of ammonia and a variety of compounds, including histidine, were supplied as sole sources of nitrogen. A revertant of strain SU1, strain 39, produced no GOGAT but high levels of nicotinamide adenine dinucleotide-dependent glutamate dehydrogenase and had restored ability to grow on a limited number of nitrogen sources. Strain US1 grew at the same rate in histidine medium as did its parent; it was derepressed for glutamine synthase synthesis, and histidase was less sensitive to repression by ammonia than in the parent strain. We conclude that GOGAT is not essential for growth on histidine but high levels of glutamine synthase are required and high levels of nicotinamide adenine dinucleotide-dependent glutamate dehydrogenase can sustain growth at low concentrations of ammonia in the absence of GOGAT.

Meers et al. (17) showed that in a variety of organisms growing in conditions of ammonia limitation, synthesis of glutamate was catalyzed by the enzyme glutamate synthase (GOGAT; L-glutamine:2-oxoglutarate aminotransferase, EC 1.4.1.13) in a reaction involving the reductive transfer of the amide nitrogen from glutamine, formed by the activity of glutamine synthetase (GS; L-glutamate:ammonia ligase, EC 6.3.1.12), to 2-oxoglutarate. The importance of this reaction as a central step in the incorporation of nitrogen from a wide variety of different sources into glutamate has been demonstrated from the properties of mutants (Asm^- strains) of *Klebsiella aerogenes*, *Klebsiella pneumoniae*, and *Escherichia coli* that lack GOGAT (1, 19, 21). Asm^- strains are still able to incorporate ammonia into glutamate if ammonia is supplied at a sufficiently high concentration, the reaction under these conditions being catalyzed by NADP-dependent glutamate dehydrogenase (NADP-GDH; GDH-L-glutamate:$NADP^+$ oxidoreductase, EC 1.4.1.4) and involving the reductive amination of 2-oxoglutarate. Asm^- strains are, however, unable to grow when ammonia is supplied at a low concentration and have, furthermore, lost the ability to grow on a wide range of compounds supplied as sole sources of nitrogen (4, 21). Failure to grow on those nitrogen sources metabolized to ammonia results presumably from the inability of the Asm^- strains to use low intracellular concentrations of ammonia for growth. However, in the presence of glucose, Asm^- strains are unable to use as nitrogen sources some compounds, such as histidine and proline, that are metabolized directly to glutamate. Prival and Magasanik (25) found that in *K. aerogenes*, the enzymes involved in the degradation of histidine (*hut*) and proline (*put*) are subject to catabolite repression by glucose when a good source of nitrogen is present in the medium. When either proline or histidine is sole source of nitrogen, growth is nitrogen limited, and under these conditions catabolite repression by glucose of the *put* or *hut* enzymes is relieved by GS, high levels of which are synthesized in conditions of nitrogen limitation (24). Failure of Asm^- mutants of *K. aerogenes* to grow on histidine or proline in the presence of glucose is attributed to their inability to synthesize levels of GS that are high enough to activate transcription of the *hut* and *put* genes. Growth of Asm^- strains under these conditions can be restored through mutations in the GlnC site which cause high levels of GS to be synthesized, even under conditions in which repression would normally be observed (4).

No detailed information is available on the role and control of the enzymes involved in the central pathway of nitrogen metabolism in *Pseudomonas aeruginosa*, but Brown et al. (6) have shown that ammonia assimilation can take place by a pathway involving GOGAT and GS and through the activity of NADP-GDH. This organism differs from *E. coli* and *K. aerogenes* in that it produces NAD-dependent glutamate de-

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hydrogenase (NAD-GDH) that is thought to be involved in the degradation of glutamate (12). Janssen et al. (12) have shown that, like other organisms, *P. aeruginosa* synthesizes high levels of non-adenylylated GS under nitrogen-limiting conditions, whereas under conditions of nitrogen excess low levels of adenylylated GS are synthesized; however, little is known about the role of GS in activating the synthesis of enzymes involved in the metabolism of individual nitrogen sources. Histidine and proline are both metabolized by the organism to yield glutamate (7, 14), the enzymes involved being induced during growth on these compounds. In *P. aeruginosa*, in contrast to the enterobacteria, succinate and other intermediates of the tricarboxylic acid cycle are generally more effective than glucose as catabolite repressors of inducible enzyme synthesis (27). The regulation of proline degradation in *P. aeruginosa* has not been investigated, but Lessie and Neidhardt (14) showed that the *hut* enzymes are subject to catabolite repression by succinate; high levels of GS have been observed in cultures grown with histidine as the growth rate-limiting source of nitrogen (20), and under these conditions no catabolite repression of histidase by succinate is observed (23). The parent strains used in the present study are defective in histidine transport such that histidine is growth rate limiting when supplied as the sole nitrogen source and cannot be used by the organism as a carbon source for growth (22).

To gain some understanding of the control of nitrogen incorporation into *P. aeruginosa*, we have studied some mutant strains lacking GOGAT through an examination of their growth properties and enzyme activities, and the results are presented in this paper.

MATERIALS AND METHODS

Bacterial strains. All strains were derived from *P. aeruginosa* strain 8602 (13). Strain AIUIN (9) carries mutations in the amidase regulator and structural genes together with an unlinked mutation responsible for increased resistance of amidase synthesis to catabolite repression (24). Strain RT1Am21 is an amidase-negative mutant derived from the acetanilide-utilizing strain RT1 and has been described previously (9).

Isolation of mutants. GOGAT-negative mutants were isolated as shadowy colonies on S-urea medium after mutagenesis of parent strains with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (3).

Spontaneous revertants of GOGAT-negative mutants were isolated as colonies growing out of a faint background of $\sim 10^8$ cells spread on S-histidine medium.

Media. Carbon sources were added to minimal salts medium (2) before autoclaving at these concentrations: 1% sodium succinate (S) and 0.3% sodium lactate. Except for urea (0.05%), nitrogen sources were at a concentration of 0.2% unless stated otherwise and, apart from ammonium sulfate (NH_4^+), were added aseptically to the medium after autoclaving. Solid media contained 1% Difco Noble agar.

Preparation of extracts. Cultures were grown at 37°C to cell densities of 3×10^8 to 4×10^8 cells per ml, and extracts were prepared as described previously (5). Protein was estimated by the method of Lowry et al. (15).

Enzyme assays. GOGAT was assayed by the method of Meers et al. (17). Optimal activity was obtained at pH 7.6. GS activity was assayed by the transferase assay essentially by the method of Stadtman et al. (28). Non-adenylylated enzyme was estimated by assaying in the presence of 60 mM $MgCl_2$; assays were performed at pH 7.8 the isoactivity point of the adenylylated and non-adenylylated enzymes (12). Rates of production of γ-glutamylhydroxamate were measured from progress curves obtained by assaying samples removed at intervals from the reaction mixture over a period of up to 15 min. NADP- and NAD-linked GDHs were measured by the methods described by Meers et al. (17). Histidase activity was measured on cell extracts by the method of Smith et al. (26). To measure glutaminase activity, extract (50 μl) was added to 2.5 ml of 5 mM γ-glutamylhydroxamate in 0.5 mM imidazole buffer (pH 7.2) at 37°C. Samples (0.5 ml each) were transferred at intervals to 1 ml of ferric chloride reagent (0.83% $FeCl_3$, 0.5 M HCl, 3% trichloroacetic acid) at 0°C. Solutions were centrifuged at 4°C and read at 500 nm against an appropriate blank.

Chemicals. γ-Glutamylhydroxamate was obtained from Sigma Chemical Co., London, England.

RESULTS AND DISCUSSION

Mutants lacking GOGAT. Two mutants strains SU1 and SU4 were isolated from strains AIU1N and 8602, respectively, as colonies growing poorly on S-urea medium. The growth properties of the mutants on solid media containing various nitrogen sources were investigated, and the results are summarized in Table 1. The inability of the mutants to utilize ammonia as a nitrogen source unless supplied at concentrations above 0.8 mM indicated a defect in a pathway specific for the incorporation of ammonia at low concentrations, and the attendant inability to use a wide range of different nitrogen sources was analogous to the behavior of Asm^- mutants of *K. aerogenes* and *E. coli* described previously (4, 21). However, Asm^- mutants of *E. coli* and *K. aerogenes* do not grow in the presence of glucose on either proline or histidine, whereas strains SU1 and SU4 grew on proline but not on histidine except to a small extent after prolonged incubation, in the presence of succinate, a strong catabolite repressor in *P. aeruginosa*. Substitution of glucose or lactate for succinate did not affect the growth properties of strains SU1 and SU4 on these two nitrogen sources. Growth on acetamide, a characteristic feature of *P. aeruginosa*, depends on the pro-

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duction of an aliphatic amidase induced by acetamide in strain 8602 but synthesized constitutively and in an altered form by strain AIU1N (9). Only after prolonged incubation was slight growth of strains SU1 and SU4 observed on medium containing acetamide as nitrogen source. Growth in liquid media reflected that observed on plates, and doubling times are included in Table 2; proline was not growth rate limiting for any of the strains.

Measurements of GOGAT activity in extracts of the parent and mutant strains grown in a variety of media showed that this enzyme was lacking in strains SU1 and SU4 (Table 2). Results for strains SU1 and SU4 grown on histidine medium are not included because periods of incubation of up to 72 h were required before significant growth was observed, and plate tests indicated that the resulting cultures contained revertants.

TABLE 1. *Utilization of different nitrogen sources by strains SU1, SU4, and their parents*

Nitrogen source	Growth[a]	
	AIU1N/8602	SU1/SU4
Ammonia (15 mM)	+	+
Ammonia (0.8 mM)	+	−
Nitrate	+	−
Nitrite	+	−
Urea	+	−
Acetamide	+	−
Glutamine	+	+
Proline	+	+
Glutamate	+	+
Histidine	+	−
Glycine	+	−
Aspartate	+	+
Tryptophan	+	−
Serine	+	−

[a] Symbols: +, single colonies visible after 24 h of incubation at 37°C on solid medium containing succinate or lactate; −, no growth.

Other enzymes of nitrogen incorporation in strains AIU, 8602, SU1, and SU4 were assayed after growth in succinate medium with ammonia, histidine, and proline supplied separately as nitrogen sources (Table 2). Substitution of lactate for succinate had no significant effect on enzyme levels, and for all the strains examined a similar general pattern was observed. For GS, the level with ammonia as the nitrogen source was the lowest, and with proline as nitrogen source, the level was five times higher. Highest levels were with histidine, which is growth rate limiting.

The high levels of GS synthesized during growth on histidine were associated with low levels of NADP-GDH. An inverse correlation between the levels of these enzymes in *P. aeruginosa* has been reported by Janssen et al. (12). Both mutant strains produced levels of NADP-GDH in S-NH_4^+ medium that were consistently two to threefold higher than that in their parent strains. This might reflect a reduction in the intracellular concentration of glutamate in the absence of GOGAT since glutamate has been found to repress NADP-GDH synthesis (12).

The levels of NAD-GDH showed no clear relationship to the levels of the other enzymes assayed. NAD-GDH is reported to be induced by glutamate and to serve a catabolic role in contrast to the biosynthetic role of NADP-GDH (12). GOGAT levels also fluctuated with the growth medium but levels on S-NH_4^+ medium were consistently high.

A revertant producing high levels of

TABLE 2. *Enzyme activities[a] of strains SU1 and SU4 and their parent strains AIU 1N and 8602 grown in media containing a variety of nitrogen sources*

Strain	Growth medium	Doubling time (min)	Sp act (U/mg of protein)				
			GOGAT	NADP-GDH	NAD-GDH	GS	
						$-Mg^{2+}$	$+Mg^{2+}$
AIU1N	S-NH_4^+ (15 mM)	52	73	81	165	65	<20
SU1			0	247	282	75	<20
8602			39	98	154	71	<20
SU4			2	161	161	ND[b]	ND
AIU1N	S-proline	52	20	16	200	547	375
SU1			0	20	200	480	281
8602			10	6	137	547	375
SU4			0	25	240	512	325
AIU1N	S-histidine	98	72	3	147	1470	590
8602			22	17	46	1080	540

[a] Enzyme units are expressed as nanomoles of product formed per minute.
[b] ND, Not determined.

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NADP-GDH. Spontaneous revertants of strains SU1 and SU4 selected on S-histidine solid medium were examined. The majority possessed the parental phenotype and were presumed to be GOGAT$^+$. Some revertants displayed a phenotype intermediate between wild type and Asm$^-$; one such strain, 39, obtained from strain SU1 grew on S-NH_4^+ medium (0.8 mM) and grew slowly on acetamide and S-urea solid media, but on all other media it resembled strain SU1 in its growth properties.

Enzyme activities of strain 39 grown in media containing a variety of nitrogen sources are shown in Table 3. The striking features of this strain were the very high level of NADP-GDH activity and an apparent GOGAT activity that was abnormally high in extracts from cells grown in proline-containing media. The apparent GOGAT activity was due to glutaminase; the high levels of NADP-GDH provided the linking enzyme in a reaction coupling the release of ammonia from glutamine to the oxidation of NADPH, giving a characteristic curved shape to the progress curve as the steady state rate was gradually approached. This interpretation was confirmed by the correlation with asparaginase activity (coordinately induced with glutaminase (16) and detected by substituting asparagine for glutamine in the assay) and by assay for γ-glutamylhydroxamate hydrolase activity. Using γ-glutamylhydroxamate as substrate (10, 11), glutaminase activity was shown to be fourfold higher in strain 39 grown with proline compared with NH_4^+ as nitrogen source. However, no significant differences in activity were found between strains 39 and SU1, and it was concluded that high NADP-GDH activity in strain 39 was responsible for the changed enzyme profile and phenotype of this revertant. Growth of strain 39 in S-histidine liquid medium was little better than strain SU1, and the level of GS was not measured.

GOGAT-negative mutant derepressed for GS. Although no revertants of strains SU1 or SU4 of the GlnC type (4) were detected on S-histidine medium, a mutant strain US1, that lacked GOGAT activity and in which GS was derepressed, was selected from strain RT1Am21 as a feebly growing colony on S-urea medium. This strain grew like the parent on S-histidine medium but lacked GOGAT activity (data not shown). Comparison of histidase and GS activities in strain US1 with the activities of its parent grown in histidine medium showed that high levels of these enzymes were maintained in strain US1 but were repressed in the parent strain when ammonium sulfate was included in the medium (Table 4). These observations supported the idea that GS activates histidase synthesis and that GOGAT activity is not essential for growth on histidine medium. Strains SU1 and SU4 may therefore be unable to grow on histidine medium through their inability to produce adequate levels of GS to activate the *hut* genes.

GOGAT seems to fulfill the same role in *P. aeruginosa* as it does in other organisms; although our results indicate that high levels of NADP-GDH can substitute for it to allow growth on some nitrogen sources. When extracts of strains SU1 and SU4 were mixed, 70% of the GOGAT activity of the parental strains was observed so that if GOGAT of *P. aeruginosa* resembles that of other organisms in comprising two nonidentical subunits (18, 29), it seems likely that the mutations affect a different GOGAT subunit in each strain. The lesion in strain US1

TABLE 4. *Comparison of histidase and GS activities of strains RT1AM21 and US1 grown in succinate medium containing histidine with and without ammonium sulfate*

Strain	Growth medium	Sp act (U/mg of protein)		
		Histidase[a]	GS[b]	
			$-Mg^{2+}$	$+Mg^{2+}$
RT1Am21	S-histidine	74	993	662
	S-histidine-NH_4^+	8	201	52
US1	S-histidine	126	1,710	1,055
	S-histidine-NH_4^+	103	1,067	632

[a] Units are nanomoles of urocanate formed per minute.
[b] Units are nanomoles of product formed per minute.

TABLE 3. *Specific activities of enzymes in extracts of strain 39 grown in various media*

Growth medium	Sp act (U[a]/mg of protein)				
	GOGAT	NADP-GDH	NAD-GDH	GS	
				$-Mg^{2+}$	$+Mg^{2+}$
S-NH_4^+	39	1,055	227	129	<20
S-proline	32	169	496	525	110
Lactate-proline	116	3,347	187	448	132
S-proline (0.05%)	358	3,258	358	617	171

[a] Units are expressed as nanomoles of product formed per minute.

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has manifold effects, and preliminary experiments indicate that intracellular concentrations of glutamine in this mutant are much higher during growth on all media than in the parent strain; there is some evidence that glutamine represses GOGAT synthesis in *B. subtilis* (8) and that this may account for the lack of GOGAT activity in strain US1. An investigation of the levels of enzymes and intermediary metabolites in partial revertants of strain US1 is currently being directed towards a more detailed characterization of this strain.

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