Regulation of Nitrogen Catabolic Enzymes in *Streptomyces clavuligerus*

By VICTORIA BASCARÁN, CARLOS HARDISSON AND
ALFREDO F. BRAÑA*

*Laboratorio de Microbiología, Departamento de Biología Funcional, Facultad de Medicina,
Universidad de Oviedo, 33006 Oviedo, Spain*

(Received 9 February 1989; revised 13 April 1989; accepted 28 April 1989)

The levels of several enzymes involved in assimilation of different nitrogen compounds were investigated in *Streptomyces clavuligerus* in relation to the nitrogen source supplied to the cultures. Threonine dehydratase, serine dehydratase, proline dehydrogenase, histidase and urocanase were not decreased in the presence of ammonium. The latter two enzymes were induced by histidine in the culture medium, while proline dehydrogenase was induced by proline. Glutamine synthetase, urease and ornithine aminotransferase levels were higher with poor nitrogen sources and were repressed by ammonium. Arginase was induced by arginine and repressed by ammonium. Glutamine synthetase was rapidly inactivated upon addition of ammonium to the culture, and could be reactivated *in vitro* by treatment with snake venom phosphodiesterase, which suggested that adenylation is involved in the inactivation. Three previously isolated mutants with abnormal glutamine synthetase activities showed pleiotropic effects on urease formation. All these data point to a mechanism controlling preferential utilization of some nitrogen sources in this species.

INTRODUCTION

The existence in bacteria of regulatory networks (regulons) that control sets of genes in response to environmental stimuli has been recognized for some time (Gottesman, 1984). Many of these regulons respond to nutritional conditions, in particular to the availability of carbon, nitrogen or phosphorus, but most of the information available deals only with enteric bacteria. The possible existence of similar mechanisms of control in members of the genus *Streptomyces* has usually been investigated in connection with antibiotic production (Martin & Demain, 1980), whose regulation may differ from that in primary metabolism. Only in the case of carbon catabolite repression is there increasing evidence for a circuit controlling utilization of different sugars (Hodgson, 1982; Seno & Chater, 1983; Fornwald *et al.*, 1987). Glucose kinase seems to play a role in this system (Ikeda *et al.*, 1984; Virolle & Bibb, 1988), which apparently differs from the regulatory mechanism found in the enteric bacteria.

The influence of nitrogen sources on secondary metabolism in *Streptomyces* is well established (Braña & Demain, 1988), but it remains to be elucidated whether there is a regulatory circuit controlling preferences in nitrogen source utilization. There are scattered reports in the literature, sometimes contradictory, on several *Streptomyces* species. For example, proline utilization was delayed in the presence of ammonium in *Streptomyces niveus* (Kominek, 1972), but not in *Streptomyces venezuelae* (Shapiro & Vining, 1985). In the latter species, ammonium prevented nitrate utilization, but no nitrogen control of nitrate reductase was observed (Shapiro & Vining, 1984). Valine dehydrogenase was repressed by ammonium in *Streptomyces fradiae* (Omura & Tanaka, 1985) but not in *Streptomyces aureofaciens* (Vancurová *et al.*, 1988);

Abbreviations: GS, glutamine synthetase; OAT, ornithine aminotransferase; SVPDE, snake venom phosphodiesterase; TCA, trichloroacetic acid.

threonine dehydratase was also repressed by ammonium in *S. fradiae* (Vancurá *et al.*, 1988). Ammonium depresses glutamine synthetase (GS; EC 6.3.1.2) activity in several *Streptomyces* (Gräfe *et al.*, 1979; Streicher & Tyler, 1981; Castro *et al.*, 1985; Braña *et al.*, 1986), but exceptions are also known (Shapiro & Vining, 1983). It is also important to remember that in most of these studies it was not determined whether inactivation of GS was responsible for the decrease in GS activity. In the present study, we have made a systematic survey of enzyme activities that might be under nitrogen control in *Streptomyces clavuligerus*, as a first step towards the elucidation of a mechanism of nitrogen regulation.

METHODS

Strains and culture conditions. Wild-type *Streptomyces clavuligerus* NRRL 3585 (ATCC 27064) was used. Three mutant strains, designated *gln-4* (lacking GS activity), *glu-2* (lacking glutamate synthase activity), and M15 (without alanine dehydrogenase), previously obtained by chemical mutagenesis (Braña *et al.*, 1986), were used in some experiments. Minimal medium for growth (MF medium) contained, per litre of water: glycerol, 10 g; K_2HPO_4 , 3.5 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; $FeSO_4 \cdot 7H_2O$, 1 mg; $MnCl_2 \cdot 4H_2O$, 1 mg; $ZnSO_4 \cdot H_2O$, 1 mg; $CaCl_2$, 1 mg (Aharonowitz & Demain, 1977). This medium was buffered at pH 6.9 with 25 mM-MOPS, adjusted with 8 M-KOH, for incubations stopped in mid-exponential phase (about 1 mg dry cell weight ml^{-1}). For incubations continued up to stationary phase, the MOPS concentration was increased to 100 mM. Nitrogen sources were used as described in the text. Only L-isomers of amino acids were used. Sporulation medium, culture conditions and growth determinations were as previously described (Braña *et al.*, 1985, 1986). For long-term preservation, spores or mycelium were lyophilized in the presence of 0.1 M-trehalose (Martín *et al.*, 1986).

Enzyme assays. *Proline dehydrogenase* (EC 1.5.99.8) was measured spectrophotometrically at 30 °C in permeabilized cells, according to Dendinger & Brill (1970). Mycelium was suspended in 0.1 M-cacodylate buffer pH 6.6 at a cell density of 9 mg dry cell weight ml^{-1} and permeabilization was achieved by one cycle of freezing (−20 °C) and rapid thawing, instead of by toluene treatment. The precipitated protein at the end of the assay was solubilized by heating at 100 °C for 15 min in 1 M-NaOH and quantified by the Lowry method.

The remaining enzyme activities were measured at 35 °C in crude extracts from cells grown to exponential phase (about 1 mg dry cell weight ml^{-1}), except where otherwise indicated. Mycelium was harvested by filtration, washed with 10 mM-MOPS pH 7.0, and used immediately or stored as a pellet at −20 °C for up to 7 d. Cells were disrupted by sonication in an ice/water bath with an MSE L50 sonifier (three 10 s pulses). Cell debris was removed by centrifugation (12000 g, 15 min, 4 °C) and the resulting cell-free extracts were placed in ice and utilized immediately.

Histidase (EC 4.3.1.3) and **urocanase** (EC 4.2.1.49) were estimated in crude extracts prepared in 50 mM-Tris/HCl pH 7.2 containing 10 mM- $MgSO_4$, 14 mM-KCl and 0.1 mM-dithiothreitol. Assays were performed as described by Kendrick & Wheelis (1982) at pH 8.5 (histidase) or 7.2 (urocanase). The procedure of Pardee & Prestidge (1955) was followed for the determination of *L-threonine dehydratase* (EC 4.2.1.16) and *L-serine dehydratase* (EC 4.2.1.13) at pH 9.5 and 6.5, respectively; *L-threonine dehydrogenase* (EC 1.1.1.103) was assayed as described by Newman *et al.* (1976). Extracts for these enzymes were prepared in 50 mM-phosphate buffer, pH 7.2. Assays for the following enzymes were made with crude extracts prepared in 10 mM-MOPS pH 7.2 containing 1 mM- $MnCl_2$. GS was assayed as γ -glutamyltransferase activity, as described previously (Braña *et al.*, 1986). **Urease** (EC 3.5.1.5) activity was measured by following the release of ammonium from urea in a reaction mixture containing 5 μ mol urea in a final volume of 0.5 ml 30 mM-phosphate buffer pH 7.3; ammonium formation was measured by the phenol/hypochlorite method (Weatherburn, 1967). The reaction mixture for the assay of **arginase** (EC 3.5.3.1) was composed of 100 μ mol L-arginine (pH 9.0) and 0.5 μ mol $MnCl_2$ in a final volume of 0.5 ml 10 mM-Tris/HCl. The reaction was stopped at intervals with 1 vol. cold 10% (w/v) trichloroacetic acid (TCA). After 30 min on ice, the precipitated protein was removed by centrifugation (12000 g, 15 min, 4 °C), and the ornithine present in the supernatant measured by high-performance liquid chromatography (HPLC) after derivatization with *o*-phthalaldehyde (Hill *et al.*, 1979). The column was a Nova-Pak C18 radial compression cartridge (0.8 × 10 cm; Waters) equilibrated with acetonitrile/12.5 mM- Na_2HPO_4 pH 7.2 (40:60, v/v). Ornithine was eluted with a linear gradient from 40% to 60% acetonitrile in 4 min, at a flow rate of 2 $ml\ min^{-1}$. No ornithine was produced in reaction mixtures lacking arginine. **Ornithine aminotransferase** (OAT, EC 2.6.1.13) was measured by spectrophotometric determination of the Δ^1 -pyrroline 5-carboxylate produced, using the assay method of Friedrich *et al.* (1978), at pH 8.0. Control reactions without 2-oxoglutarate were run in parallel. **Maltase** (EC 3.2.1.20) was determined according to Zimmerman & Eaton (1974). The pH was optimized in all the assays except in the case of maltase and proline dehydrogenase. A unit (U) of activity is defined as the amount of enzyme that produces 1 μ mol product min^{-1} under standard assay conditions. Activities were normalized by the protein content of the crude extracts or permeabilized cells, measured by the Lowry method. The values given in Tables are the means of at least two independent experiments. Standard deviations were within 15% of the mean values represented.

Reactivation of GS. Crude extracts from cells grown to exponential phase, with or without previous ammonium shock (Braña *et al.*, 1986), were prepared in 20 mM-imidazole/HCl pH 7.3, containing 2.5 mM-MnCl₂. The extracts were incubated at 35 °C with snake venom phosphodiesterase (SVPDE, Boehringer Mannheim) at 100 µg ml⁻¹, and the GS activity determined at intervals (Streicher & Tyler, 1981).

Amino acid analysis. Residual amino acids in the culture media were determined by HPLC (Hill *et al.*, 1979). Proline, which is not derivatized by this method, was measured colorimetrically as described by Shapiro & Vining (1983). To extract the intracellular pool of free amino acids, a volume of culture containing 20 mg cells (dry cell weight) was filtered and washed with 10 mM-MOPS pH 7.0. The filter with the cells was immediately immersed in 1 ml methanol and stored at -20 °C. Extraction was completed with TCA as described by Braña *et al.* (1986) and the amino acids determined by HPLC.

RESULTS

Reactivation of GS

The GS of *S. clavuligerus* is rapidly inactivated when ammonium is added to a derepressed culture (Braña *et al.*, 1986). When a crude extract prepared from ammonium-shocked cells was incubated with SVPDE, GS activity increased up to the level of a non-shocked control extract (Fig. 1). A slow spontaneous reactivation was observed during incubation in the absence of SVPDE. Previous heating of the extracts at 60 °C for 30 min did not affect GS activity, but it eliminated spontaneous reactivation, although GS could still be reactivated normally by later incubation with SVPDE. Thus, the spontaneous reactivation was likely to be due to some endogenous enzyme activity that is destroyed by the heat treatment.

Crude extracts obtained from exponential-phase cultures growing with different single nitrogen sources were treated with SVPDE to investigate the extent of GS modification during growth in each case. Extracts from ammonium-shocked cells were used in parallel to ensure that the reaction had been complete. The degree of reactivation obtained was variable, depending on the nitrogen source, ranging from a 21% increase in GS activity (with NH₄Cl) to negligible reactivation (with proline).

Nitrogen control in wild-type *S. clavuligerus*

The regulation of several enzymes involved in the catabolism of nitrogen sources was examined in relation to the composition of the culture medium. Proline dehydrogenase activity was detected in cells permeabilized by freezing and thawing. Other permeabilizing treatments (toluene, acetone, hexadecyltrimethylammonium bromide) or the use of intact cells or crude extracts resulted in much lower proline dehydrogenase activities. As shown in Table 1, proline

Fig. 1. Reactivation of GS with SVPDE. A culture of *S. clavuligerus* growing with glutamate as nitrogen source was divided into two parts and 20 mM-NH₄Cl added to one. After 30 min incubation, both cultures were harvested and crude extracts prepared. The extracts of the ammonium-shocked (Δ) and unshocked (○) cultures were incubated with SVPDE and the GS activity assayed at intervals. As a control, an extract of the shocked culture was also incubated in the absence of SVPDE (▲). The kinetics of reactivation were very reproducible for each batch of SVPDE. A representative result is shown.

Fig. 2. Nitrogen source consumption in relation to growth of *S. clavuligerus*. Cells were cultured in defined medium containing 40 mM- NH_4Cl plus 30 mM-proline (a), threonine (b) or arginine (c). Residual ammonium (●) or amino acids (△) in the culture medium were determined as described in Methods. The experiment was done in duplicate and the extrapolated concentration of residual nitrogen source at any given dry cell weight had SD values lower than 8% of the mean. The data shown are from one experiment.

Table 1. Regulation of proline dehydrogenase in *S. clavuligerus*

Nitrogen source*	Proline dehydrogenase [mU (mg protein) ⁻¹]
Glutamate	0.11
Alanine	0.14
Proline	1.29
Proline + NH_4Cl	1.21
Proline + NH_4Cl + Glutamine	0.95

* Glutamate, alanine and proline were used at 30 mM, glutamine at 15 mM and NH_4Cl at 40 mM.

Table 2. Regulation of histidine catabolic enzymes in *S. clavuligerus*

Nitrogen source*	Specific activity [mU (mg protein) ⁻¹]	
	Histidase	Urocanase
Alanine	1.0	14.7
Glutamine	1.6	12.0
Histidine	41.0	45.3
Histidine + NH_4Cl	42.1	45.4
Histidine + Glutamine	31.0	36.4
Histidine + Alanine	37.7	33.6

* Amino acids were used at a concentration providing 30 mM nitrogen. NH_4Cl was added at 40 mM.

dehydrogenase levels increased when proline was present in the culture medium, even in the presence of ammonium or glutamine plus ammonium. Furthermore, ammonium did not prevent utilization of proline when the cells were grown with a mixture of these two nitrogen sources (Fig. 2a). Both histidase and urocanase were induced in the presence of histidine, either as a sole nitrogen source or in combination with other nitrogen compounds, including glutamine or ammonium (Table 2).

Two enzyme activities, threonine dehydratase and serine dehydratase, involved in hydroxy amino acid metabolism, were detected in crude extracts, while threonine dehydrogenase activity

Table 3. Regulation of threonine dehydratase and serine dehydratase in *S. clavuligerus*

Nitrogen source*	Specific activity [mU (mg protein) ⁻¹]	
	Threonine dehydratase	Serine dehydratase
Threonine	1.75	1.96
Threonine + NH ₄ Cl	5.77	5.47
NH ₄ Cl	4.05	2.76

* Threonine was used at 30 mM and NH₄Cl at 40 mM.

Table 4. Regulation of urease and GS in wild-type and mutant strains of *S. clavuligerus*

Nitrogen source*	Specific activity [mU (mg protein) ⁻¹] in:					
	Wild-type		M15		<i>glu-2</i>	
	Urease	GS	Urease	GS	Urease	GS
Glutamine	< 10	391	182	3022	< 10	254
NH ₄ Cl	< 10	499	< 10	597	ND	ND
Asparagine	< 10	431	246	2909	ND	ND
Aspartate	< 10	595	492	2909	ND	ND
Urea	36	567	ND	ND	ND	ND
Glutamate	232	3837	400	2488	< 10	547
Alanine	244	2149	ND	ND	ND	ND
Threonine	298	3634	ND	ND	ND	ND
Arginine	404	2086	ND	ND	ND	ND
Proline	514	3063	ND	ND	262	1617
Glutamate + NH ₄ Cl	< 10	602	ND	ND	ND	ND
Alanine + NH ₄ Cl	36	653	ND	ND	ND	ND

ND, Not determined.

* NH₄Cl supplements were added at 40 mM. All other nitrogen sources were used at a concentration providing 30 mM nitrogen.

could not be found. These enzymes were not inhibited *in vitro* by L-isoleucine at concentrations up to 10 mM, which suggested their catabolic role. However, the activities found in media with threonine as a nitrogen source (no growth was observed in liquid MF medium with serine as sole nitrogen source) were not lowered when the medium was supplemented with ammonium (Table 3). In fact, higher activities were found in the presence of ammonium, even when it was provided as the only source of nitrogen. Furthermore, threonine and NH₄Cl were utilized simultaneously during growth in MF medium with a mixture of these compounds (Fig. 2b), confirming that ammonium does not prevent threonine catabolism.

Determination of GS and urease levels in extracts of cells growing with a variety of nitrogen sources showed two well-differentiated situations (Table 4). When the cells were incubated with nitrogen sources that resulted in low GS activities, which may be interpreted as nitrogen-excess conditions, the corresponding urease levels were very low. Conversely, those cases where GS activity was high correlated with high urease activity. Addition of ammonium to media supporting high GS values resulted in low activities of both enzymes.

The activities of two enzymes involved in arginine catabolism, arginase and OAT, were also influenced by the nature of the nitrogen source employed (Table 5). Although low levels of OAT were always found, the activity of this enzyme increased in cultures growing with nitrogen sources that resulted in high GS levels. Among the nitrogen compounds tested, arginase increased only with arginine. Addition of ammonium or glutamine plus ammonium reversed the induction effect of arginine and decreased OAT activity to the basal level. The possibility that the ammonium effect could be due to a phenomenon of inducer-exclusion was tested by

Table 5. *Regulation of arginase and OAT in S. clavuligerus*

Nitrogen source*	Specific activity [mU (mg protein) ⁻¹]			Intracellular arginine [nmol (mg dry cell wt) ⁻¹]
	Arginase	OAT	GS	
NH ₄ Cl	0.31	0.93	437	ND
Aspartate	0.53	0.84	515	ND
Threonine	0.61	3.45	2246	ND
Alanine	0.61	3.73	2410	3.7
Arginine	2.08	3.30	2362	59.5
Arginine + NH ₄ Cl	0.90	1.16	847	46.0
Arginine + Glutamine + NH ₄ Cl	0.64	1.03	681	42.3

ND, Not determined.

* NH₄Cl supplements were added at 40 mM. All other nitrogen sources were used at a concentration providing 30 mM nitrogen.

Table 6. *Formation of urease upon nitrogen starvation in S. clavuligerus*

Cells growing in medium with 1% (w/v) glycerol, 15 mM-glutamine and 40 mM-NH₄Cl were collected in the exponential phase. Part of the mycelium was resuspended in medium lacking nitrogen source, with 1% (w/v) glycerol or maltose as carbon source, and incubated for 15 h.

Carbon source	Nitrogen source	Specific activity [mU (mg protein) ⁻¹]			
		Wild-type		<i>gln-4</i>	
		Urease	Maltase	Urease	Maltase
Glycerol	Glutamine + NH ₄ Cl	< 10	0.8	< 10	1.6
Glycerol	None	464	ND	< 10	ND
Maltose	None	110	20.8	< 10	35.0

ND, Not determined.

measuring the intracellular content of arginine in cells grown with different nitrogen sources (Table 5). A high intracellular pool of arginine was found whenever arginine was used in the medium. Ammonium or glutamine plus ammonium slightly reduced the arginine pool but the values obtained in these cases were well above that of a control culture without arginine. Thus, it seems likely that ammonium did not severely impair the entry of arginine into the cells. In cultures supplied with a mixture of ammonium and arginine, the former was used preferentially, with very little arginine being catabolized (Fig. 2c). Both types of evidence thus suggest a nitrogen control of arginine utilization.

Cells growing in MF medium with arginine plus glutamate plus ammonium had low activities of the four nitrogen-controlled enzymes (GS, urease, arginase and OAT). Upon transfer of these cells to MF medium with arginine as the sole nitrogen source, all four activities increased (measured after 5 h of incubation in the new medium), but this increase was prevented by chloramphenicol (100 µg ml⁻¹) or rifampicin (15 µg ml⁻¹) (data not shown), suggesting that the formation of these enzymes was nitrogen-repressed at the transcriptional level.

Nitrogen control in S. clavuligerus mutants

Using GS and urease as a model of nitrogen-controlled enzymes, we have investigated the regulatory behaviour of three mutants of *S. clavuligerus* with alterations in ammonium assimilation. The M15 strain, devoid of significant alanine dehydrogenase activity, produces high GS levels even in repressive media, except when ammonium is the nitrogen source (Braña *et al.*, 1986). When urease activities were measured in this mutant, a coordinate derepression of

GS and urease was observed with most nitrogen sources that were repressive for the wild-type (Table 4).

The glutamate-synthase-deficient strain *glu-2* showed low GS and urease values during growth with glutamate, but both activities were increased if a very poor nitrogen source, such as proline, was employed.

The glutamine auxotroph *gln-4*, devoid of GS activity, was unable to derepress urease, not only during growth in the presence of glutamine, but even after prolonged nitrogen starvation that caused urease synthesis in the wild-type (Table 6). In order to distinguish whether the lack of urease formation was due to a regulatory phenomenon or to an impairment in protein synthesis caused by the glutamine requirement, nitrogen starvation was also imposed in the presence of maltose. Under these conditions, both the wild type and *gln-4* could induce maltase, but urease remained repressed in *gln-4* (Table 6).

DISCUSSION

The previously observed dependence of GS formation on the available nitrogen source and its repression by ammonium was complicated by the simultaneous finding of a mechanism of GS inactivation (Braña *et al.*, 1986). To make sure that the effect of nitrogen was mainly exerted on enzyme formation, and not on its activity, we tried to fully reactivate the enzyme in crude extracts in order to obtain more reliable values of GS concentration. SVPDE treatment resulted in complete recovery of activity in crude extracts of ammonium-shocked cells, which indicates that GS inactivation is mediated by covalent modification. Adenylylation is the most likely type of modification, as has been described in many bacteria, including *Streptomyces* (Streicher & Tyler, 1981; Xia & Jiao, 1986), although the possibility of a different type of modification involving a phosphodiester linkage cannot be excluded by our data. The relatively reduced levels of modification found during exponential growth with different nitrogen sources (up to 21% increase in activity after SVPDE treatment) cannot explain the broad variations in GS activity. Therefore, these changes are likely to reflect differences in enzyme synthesis, as suggested for *Streptomyces cattleya* by Parese & Streicher (1985).

In addition to GS, we have studied a variety of nitrogen compounds whose catabolism has often been found to be under nitrogen control in other bacteria. For example, ammonium repression of the catabolic enzymes involved in proline utilization has been described (Tyler, 1978; Meile *et al.*, 1982). *S. clavuligerus* did not behave in this way, as ammonium neither prevented proline consumption, nor repressed the activity of proline dehydrogenase.

Histidase and urocanase activities were detected in crude extracts of *S. clavuligerus*, and their induction by histidine suggested a role in the catabolism of this amino acid. These enzymes, particularly histidase, are among the best-known examples of nitrogen regulation in *Klebsiella aerogenes* (*K. pneumoniae*), where they are induced in the presence of histidine and repressed by ammonium (Tyler, 1978). No such repression was found in *S. clavuligerus*, in accordance with previous observations in *S. coelicolor* (Kendrick & Wheelis, 1982) and *S. griseus* (Kroening & Kendrick, 1987), indicating that nitrogen control is not involved in histidine utilization in these species.

The utilization of threonine as sole nitrogen source by *S. clavuligerus* may occur by deamination and subsequent assimilation of the resulting ammonium, as suggested by the absence of growth with threonine in mutants lacking glutamate synthase (Braña *et al.*, 1986). At least one enzyme activity, threonine dehydratase, which can carry out that deamination, was detected in crude extracts, together with a similar activity acting on serine. No indication of ammonium repression was found with either of these enzymes and, although it is unclear whether the threonine dehydratase activity represents the actual pathway of threonine catabolism, the data on nitrogen source consumption ruled out a strong effect of ammonium on the utilization of threonine. It should be mentioned, however, that interspecific differences are likely to occur, as threonine dehydratase has been reported to be ammonium-repressed in *S. fradiae* (Vancurá *et al.*, 1988).

Arginine is used as a nitrogen source by wild-type *S. clavuligerus* and mutants devoid of glutamate synthase (Braña *et al.*, 1986). These mutants, however, failed to grow on arginine in the presence of ammonium (data not shown), which may be interpreted as evidence of nitrogen control of arginine degradation (Pahel *et al.*, 1978). Indeed, ammonium interfered with arginine utilization, and at least two enzymes involved in the catabolism of this amino acid are regulatory targets. Arginase activity has been reported in *S. clavuligerus* and found to be induced by arginine (Romero *et al.*, 1986). Our results confirmed this observation and also indicated that nitrogen control participates in arginase regulation, as described in other micro-organisms (Baumberg & Harwood, 1979; Broman *et al.*, 1978). The next enzyme in the arginase pathway, OAT, is also arginine-induced and controlled by nitrogen in some bacteria (Baumberg & Harwood, 1979; Friedrich *et al.*, 1978). In *S. clavuligerus* this enzyme was also found to be controlled according to the nitrogen status of the cells, reflected by the GS levels, but no induction by arginine was observed.

Regulation of urease formation follows a characteristic pattern in many micro-organisms: urea is not required as an inducer and the enzyme is produced at high levels during nitrogen-limited growth but repressed under conditions of nitrogen excess (Friedrich & Magasanik, 1977; Janssen *et al.*, 1981; Kaltwasser *et al.*, 1972; Patterson & Hespell, 1985; Smith *et al.*, 1981). This was the behaviour observed in *S. clavuligerus*, where urease levels exhibited dramatic changes as a function of the nitrogen source.

The correlation observed between GS and the levels of some nitrogen catabolic enzymes in *S. clavuligerus* prompted us to study the behaviour of mutants altered in the formation of GS. A modified control of those enzymes would be expected if a regulatory relationship with GS existed. This was confirmed first with mutant M15, where the apparent pleiotropic derepression of GS caused by the alanine dehydrogenase mutation (Braña *et al.*, 1986) was accompanied by a simultaneous derepression of urease. The coordination was maintained in the strain *glu-2*, while in the glutamine auxotroph strain *gln-4* the lack of GS activity was also correlated with the inability to derepress urease, even after nitrogen starvation. It is interesting to note that in bacteria known to possess systems of nitrogen control, some mutations cause a particular phenotype (usually known as *Ntr*⁻) characterized by the inability to express enzyme activities controlled by nitrogen and, very often, a glutamine requirement (Magasanik, 1982; Merrick, 1988), which resembles the characteristics of the strain *gln-4*.

The results obtained with *S. clavuligerus* indicated that the activities of at least four enzymes involved in nitrogen nutrition are controlled according to the nitrogen source supplied to the cultures and all four are decreased by ammonium. The regulation of GS and urease changed coordinately in three ammonium-assimilation mutants, which can hardly be explained by the presence of multiple mutations in every case. Therefore, a system of nitrogen control linked to the pathway of assimilation of ammonium, a common phenomenon in Gram-negative bacteria but virtually unknown in Gram-positive micro-organisms, may exist in this species. Further evidence that reinforces this hypothesis is provided in the accompanying paper (Bascarán *et al.*, 1989).

In preliminary observations in *S. coelicolor* A3(2) we have found that neither arginase nor nitrate reductase is ammonium-repressed, but GS and urease activities changed moderately as a function of the nitrogen source (unpublished results), which agrees with a recent report on this species (Wray & Fisher, 1988). A defect in nitrogen assimilation that might be related to nitrogen control has been reported in a closely related strain, *S. lividans*, in association with genetic instability (Dyson & Schrempf, 1987). These data, taken together with the observations in other strains mentioned in the Introduction, suggest that a mechanism of nitrogen control may be common in streptomycetes. However, even among bacteria that share the *ntr* system of nitrogen control, there is variation in the regulation of particular pathways involved in the catabolism of nitrogen compounds (Merrick, 1988), and such variation is likely to occur in streptomycetes also.

REFERENCES

- AHARONOWITZ, Y. & DEMAINE, A. L. (1977). Influence of inorganic phosphate and organic buffers on cephalosporin production by *Streptomyces clavuligerus*. *Archives of Microbiology* **115**, 169–173.
- BASCARÁN, V., HARDISSON, C. & BRAÑA, A. F. (1989). Isolation and characterization of nitrogen-deregulated mutants of *Streptomyces clavuligerus*. *Journal of General Microbiology* **135**, 2475–2482.
- BAUMBERG, S. & HARWOOD, C. R. (1979). Carbon and nitrogen repression of arginine catabolic enzymes in *Bacillus subtilis*. *Journal of Bacteriology* **137**, 189–196.
- BRAÑA, A. F. & DEMAINE, A. L. (1988). Nitrogen control of antibiotic biosynthesis in actinomycetes. In *Nitrogen Source Control of Microbial Processes*, pp. 99–119. Edited by S. Sánchez-Esquivel. Boca Ratón, Fla.: CRC Press.
- BRAÑA, A. F., WOLFE, S. & DEMAINE, A. L. (1985). Ammonium repression of cephalosporin production by *Streptomyces clavuligerus*. *Canadian Journal of Microbiology* **31**, 736–743.
- BRAÑA, A. F., PAIVA, N. & DEMAINE, A. L. (1986). Pathways and regulation of ammonium assimilation in *Streptomyces clavuligerus*. *Journal of General Microbiology* **132**, 1305–1317.
- BROMAN, K., LAUWERS, N., STALON, V. & WIAME, J. M. (1978). Oxygen and nitrate in utilization by *Bacillus licheniformis* of the arginase and arginine deiminase routes of arginine catabolism and other factors affecting their synthesis. *Journal of Bacteriology* **135**, 920–927.
- CASTRO, J. M., LIRAS, P., CORTES, J. & MARTIN, J. F. (1985). Regulation of α -amino acid synthetase, isopenicillin N synthetase, isopenicillin N isomerase and deacetoxycephalosporin C synthetase by nitrogen sources in *Streptomyces lactamdurans*. *Applied Microbiology and Biotechnology* **21**, 32–40.
- DENDINGER, S. & BRILL, W. J. (1970). Regulation of proline degradation in *Salmonella typhimurium*. *Journal of Bacteriology* **103**, 144–152.
- DYSON, P. & SCHREMPF, H. (1987). Genetic instability and DNA amplification in *Streptomyces lividans* 66. *Journal of Bacteriology* **169**, 4796–4803.
- FORNWALD, J. A., SCHMIDT, F. J., ADAMS, C. W., ROSENBERG, M. & BRAWNER, M. E. (1987). Two promoters, one inducible and one constitutive, control transcription of the *Streptomyces lividans* galactose operon. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 2130–2134.
- FRIEDRICH, B. & MAGASANIK, B. (1977). Urease of *Klebsiella aerogenes*: control of its synthesis by glutamine synthetase. *Journal of Bacteriology* **131**, 446–452.
- FRIEDRICH, B., FRIEDRICH, C. G. & MAGASANIK, B. (1978). Catabolic N^2 -acetylornithine 5-aminotransferase of *Klebsiella aerogenes*: control of synthesis by induction, catabolite repression, and activation by glutamine synthetase. *Journal of Bacteriology* **133**, 686–691.
- GOTTESMAN, S. (1984). Bacterial regulation: global regulatory networks. *Annual Review of Genetics* **18**, 415–441.
- GRÄFE, U., BOCKER, H. & THURM, H. (1979). Regulation der Glutamin-synthetasebildung in *Streptomyces*. *Zeitschrift für allgemeine Mikrobiologie* **19**, 663–666.
- HILL, D. W., WALTERS, F. H., WILSON, D. & STUART, J. D. (1979). High performance liquid chromatographic determination of amino acids in the picomole range. *Analytical Chemistry* **51**, 1338–1341.
- HODGSON, D. A. (1982). Glucose repression of carbon source uptake and metabolism in *Streptomyces coelicolor* A3(2) and its perturbation in mutants resistant to 2-deoxyglucose. *Journal of General Microbiology* **128**, 2417–2430.
- IKEDA, H., SENO, E. T., BRUTON, C. J. & CHATER, K. F. (1984). Genetic mapping, cloning and physiological aspects of the glucose kinase gene of *Streptomyces coelicolor*. *Molecular and General Genetics* **196**, 501–507.
- JANSEN, D. B., HERST, P. M., JOOSTEN, H. M. L. J. & VAN DER DRIFT, C. (1981). Nitrogen control in *Pseudomonas aeruginosa*: a role for glutamine in the regulation of the synthesis of NADP-dependent glutamate dehydrogenase, urease and histidase. *Archives of Microbiology* **128**, 398–402.
- KALTWASSER, H., KRAMER, J. & CONGER, W. R. (1972). Control of urease formation in certain aerobic bacteria. *Archives of Microbiology* **81**, 178–196.
- KENDRICK, K. E. & WHEELIS, M. L. (1982). Histidine dissimilation in *Streptomyces coelicolor*. *Journal of General Microbiology* **128**, 2029–2040.
- KOMINEK, L. A. (1972). Biosynthesis of novobiocin by *Streptomyces niveus*. *Antimicrobial Agents and Chemotherapy* **1**, 123–134.
- KROENING, T. A. & KENDRICK, K. E. (1987). *In vivo* regulation of histidine ammonia-lyase activity from *Streptomyces griseus*. *Journal of Bacteriology* **169**, 823–829.
- MAGASANIK, B. (1982). Genetic control of nitrogen assimilation in bacteria. *Annual Review of Genetics* **16**, 135–168.
- MARTIN, J. F. & DEMAINE, A. L. (1980). Control of antibiotic biosynthesis. *Microbiological Reviews* **44**, 230–251.
- MARTÍN, M. C., DIAZ, L. A., MANZANAL, M. B. & HARDISSON, C. (1986). Role of trehalose in the spores of *Streptomyces*. *FEMS Microbiology Letters* **35**, 49–54.
- MEILE, L., SOLDATI, L. & LEISINGER, T. (1982). Regulation of proline catabolism in *Pseudomonas aeruginosa* PAO. *Archives of Microbiology* **132**, 189–193.
- MERRICK, M. J. (1988). Regulation of nitrogen assimilation by bacteria. In *The Nitrogen and Sulphur Cycles*, pp. 331–361. Edited by J. A. Cole & S. J. Ferguson. Cambridge: Cambridge University Press.
- NEWMAN, E. B., KAPOOR, V. & POTTER, R. (1976). Role of L-threonine dehydrogenase in the catabolism of threonine and synthesis of glycine by *Escherichia coli*. *Journal of Bacteriology* **126**, 1245–1249.
- OMURA, S. & TANAKA, Y. (1985). Biosynthesis of tylosin and its regulation by ammonium and phosphate. In *Regulation of Secondary Metabolite Formation*, pp. 305–332. Edited by H. Kleinkauf, H. von Döhren, H. Dornauer & G. Nesemann. Weinheim, FRG: VCH Verlagsgesellschaft.

- PAHEL, G., ZELENETZ, A. D. & TYLER, B. (1978). *glbB* gene and regulation of nitrogen metabolism by glutamine synthetase in *Escherichia coli*. *Journal of Bacteriology* **133**, 139–148.
- PARDEE, A. B. & PRESTIDGE, L. S. (1955). Induced formation of serine and threonine deaminases by *Escherichia coli*. *Journal of Bacteriology* **70**, 667–674.
- PARESS, P. S. & STREICHER, S. L. (1985). Glutamine synthetase of *Streptomyces cattleya*: purification and regulation of synthesis. *Journal of General Microbiology* **131**, 1903–1910.
- PATTERSON, J. A. & HESPELL, R. B. (1985). Glutamine synthetase activity in the ruminal bacterium *Succinivibrio dextrinosolvens*. *Applied and Environmental Microbiology* **50**, 1014–1020.
- ROMERO, J., LIRAS, P. & MARTIN, J. F. (1986). Utilization of ornithine and arginine as specific precursors of clavulanic acid. *Applied and Environmental Microbiology* **52**, 892–897.
- SENO, E. T. & CHATER, K. F. (1983). Glycerol catabolic enzymes and their regulation in wild-type and mutant strains of *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* **129**, 1403–1413.
- SHAPIRO, S. & VINING, L. C. (1983). Nitrogen metabolism and chloramphenicol production in *Streptomyces venezuelae*. *Canadian Journal of Microbiology* **29**, 1706–1714.
- SHAPIRO, S. & VINING, L. C. (1984). Suppression of nitrate utilization by ammonium and its relationship to chloramphenicol production in *Streptomyces venezuelae*. *Canadian Journal of Microbiology* **30**, 798–804.
- SHAPIRO, S. & VINING, L. C. (1985). Effect of ammonium on chloramphenicol production by *Streptomyces venezuelae*. *Canadian Journal of Microbiology* **31**, 119–123.
- SMITH, C. J., HESPELL, R. B. & BRYANT, M. P. (1981). Regulation of urease and ammonia assimilatory enzymes in *Selenomonas ruminantium*. *Applied and Environmental Microbiology* **42**, 89–96.
- STREICHER, S. L. & TYLER, B. (1981). Regulation of glutamine synthetase activity by adenylation in the Gram-positive bacterium *Streptomyces cattleya*. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 229–233.
- TYLER, B. (1978). Regulation of the assimilation of nitrogen compounds. *Annual Review of Biochemistry* **47**, 1127–1162.
- VANCURÁ, A., REZANKA, T., MARSLALEK, J., MELZUCH, K., BASAROVA, G. & KRISTAN, V. (1988). Metabolism of L-threonine and fatty acids and tylosin biosynthesis in *Streptomyces fradiae*. *FEMS Microbiology Letters* **49**, 411–415.
- VANCUROVÁ, I., VANCURA, A., VOLC, J., NEUZIL, J., FLIEGER, M., BASAROVA, G. & BEHAL, V. (1988). Isolation and characterization of valine dehydrogenase from *Streptomyces aureofaciens*. *Journal of Bacteriology* **170**, 5192–5196.
- VIROLLE, M. J. & BIBB, M. J. (1988). Cloning, characterization and regulation of an α -amylase gene from *Streptomyces limosus*. *Molecular Microbiology* **2**, 197–208.
- WEATHERBURN, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry* **39**, 971–974.
- WRAY, L. V. & FISHER, S. H. (1988). Nitrogen regulation in *Streptomyces coelicolor*. *7th International Symposium on Biology of Actinomycetes* (May 1988, Tokyo, Japan), abstract S1a-3.
- XIA, T. & JIAO, R. S. (1986). Studies on glutamine synthetase from *Streptomyces hygroscopicus* var. *jinggangensis*. *Scientia sinica (Series B)* **29**, 379–388.
- ZIMMERMANN, F. K. & EATON, N. R. (1974). Genetics of induction and catabolite repression of maltase synthesis in *Saccharomyces cerevisiae*. *Molecular and General Genetics* **134**, 261–272.