The Effect of Detergents on Amino Acid Liberation by the N₂-fixing Cyanobacterium *Anabaena variabilis* and 6-Fluorotryptophan-resistant Mutant Strains

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The effects of the polyoxyethylene stearate detergents MYRJ 45 and MYRJ 52 on FT-9, an amino-acid-liberating mutant strain of *Anabaena variabilis*, and on the parent strain were investigated. Both strains were capable of growth in the presence of these detergents, which induced the liberation of amino acids by the parent strain and increased the liberation of amino acids by the mutant strain. Some changes in the spectrum of amino acids liberated by the mutant strain were observed in the presence of detergents, notably the liberation of tryptophan, which was not detected in the absence of detergents.

INTRODUCTION

Certain cyanobacteria can fix N₂ in addition to fixing carbon photosynthetically, and can therefore grow in the absence of organic carbon and combined nitrogen. We have exploited this property by isolating mutant strains of the N₂-fixing cyanobacterium *Anabaena variabilis* which overproduce and liberate amino acids when grown in simple inorganic media (Kerby *et al.*, 1987). The mutant strains were immobilized in calcium alginate gels and the feasibility of this system for the continuous production of amino acids was demonstrated.

The mutant strains were selected for resistance to the toxic tryptophan analogue 6-fluorotryptophan, but none liberated detectable quantities of tryptophan. One liberated predominantly aromatic amino acids (phenylalanine and tyrosine), but most liberated approximately 50% of the total amino acids produced as alanine, with phenylalanine and tyrosine together forming less than 10% of the total (Kerby *et al.*, 1987; our unpublished data). However, all the mutants investigated appear to have acquired their resistance to the analogue at least partly through a deregulation of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHP synthase), the first enzyme of the pathway for the biosynthesis of aromatic amino acids (unpublished data).

The extracellular liberation of a product by a micro-organism requires either that the cell membrane is freely permeable to that product or that the product is actively transported out of the cell. In many industrial processes for the production of metabolites by micro-organisms it is necessary to facilitate product liberation by altering the permeability of the plasma membrane by, for example, growth factor deficiency, antibiotic treatment or the addition of detergents (Fukui & Ishida, 1972). The excretion of glutamate by *Corynebacterium glutamicum* is achieved industrially by the addition of an acylated surfactant to the culture (Demain & Birnbaum, 1968). Prior to surfactant addition, this strain liberated only alanine. The surfactant has been shown to

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Abbreviations: chl *a*, chlorophyll *a*; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; HLB, hydrophilic-lipophilic balance.

cause loss of membrane phospholipids which results in an uncoupling of the glutamate uptake system, thus facilitating the release of glutamate from the cell (Clément *et al.*, 1984; Clément & Lanéeelle, 1986).

To date, very little work has been done on the effects of detergent treatment on cyanobacteria. It was the purpose of this study to investigate such effects and to determine whether detergents may be used to alter the types and quantities of amino acids liberated by mutant strains of *A. variabilis*.

METHODS

Organisms and growth conditions. *Anabaena variabilis* Kütz. (ATCC 29413) was grown in 100 ml batch cultures in BG11₀ medium (Rippka *et al.*, 1979) incubated at 21 °C on an orbital shaker. Illumination was provided by cool white fluorescent tubes at a photon flux density of 40 $\mu\text{E m}^{-2} \text{s}^{-1}$ incident at the surface of the vessels. Strain FT-9 was selected for resistance to 6-fluorotryptophan (40 $\mu\text{g ml}^{-1}$) after chemical mutagenesis (Kerby *et al.*, 1987).

Chlorophyll *a* (chl *a*) determination. Chlorophyll was extracted in methanol in the dark at 4 °C for 18 h. Absorbance was measured at 663 nm and the chl *a* concentration was calculated according to Mackinney (1941).

Photosynthetic O₂ evolution. Samples of culture were placed in a Rank Pt-Ag electrode at 30 °C with saturating light provided by a tungsten filament lamp. The value obtained was the sum of O₂ evolved during illumination and O₂ consumed in the dark.

Nitrogenase activity. This was determined by the acetylene reduction method of Stewart *et al.* (1967). Portions (1 ml) of culture were placed in 7 ml bottles with 10% (v/v) acetylene in air as the gas phase. Incubation was at 21 °C with a photon flux density of 40 $\mu\text{E m}^{-2} \text{s}^{-1}$.

Amino acid analysis. A ninhydrin colorimetric assay was used to estimate the total amino acids liberated into the medium (Rosen, 1957). Analyses of the individual amino acids in the media were done using an LKB 4400 amino acid analyser and a lithium citrate buffer system as described by Kerby *et al.* (1987).

Detergents. MYRJ 45 and MYRJ 52 were produced by Serva Feinbiochemica and obtained from Uniscience.

RESULTS

The effects of two polyoxyethylene stearate detergents, MYRJ 45 and MYRJ 52, on an amino-acid-liberating mutant of *A. variabilis* (FT-9) and on the parent strain were investigated.

Both the parent strain and FT-9 were capable of growth in BG11₀ medium, under the conditions used, with up to 0.5% (w/v) detergent, which was the most concentrated solution which could be used in liquid culture (Fig. 1). With MYRJ 45, the growth of both strains was reduced at the higher detergent concentrations. In contrast, MYRJ 52 either had very little effect or caused a slight stimulation of growth of FT-9, depending on the concentration used. When applied to the parent strain, MYRJ 52 inhibited growth at 0.5% (w/v) but otherwise had little effect.

The rates of photosynthetic O₂ evolution and C₂H₂ reduction were measured in cultures grown for 14 d in medium containing 0.1% (w/v) detergent (Table 1). The mutant strain had a lower rate of photosynthetic O₂ evolution than the parent strain but the rates for those cultures grown in the presence of detergent were similar to the controls in each case. FT-9 had a higher rate of C₂H₂ reduction than the parent strain in control cultures and MYRJ 45 and MYRJ 52 decreased the rate in both strains to a similar extent.

The total amino acids liberated into the medium by detergent-grown batch cultures of FT-9 and the parent strain were measured at intervals over several weeks (Fig. 2). Only traces of ninhydrin-positive material were detected in control cultures of the parent strain and this was slightly increased in the presence of MYRJ 45. MYRJ 52 caused a substantial liberation of amino acids by the parent strain when used at a concentration of 0.5% (w/v). However, in view of the slower growth of the parent strain in the presence of 0.5% (w/v) MYRJ 52 (Fig. 1), amino acid release may have resulted partly from cell lysis. FT-9 liberated amino acids into the medium in the absence of detergents but MYRJ 52 substantially increased amino acid liberation, the most effective concentration being 0.5% (w/v). MYRJ 45 caused little or no increase in amino acid liberation by FT-9.

Amino acid productivity per unit biomass (chl *a*) in the parent strain, in the presence of 0.1% (w/v) of either detergent, reached a maximum approximately 7 d after inoculation and subsequently decreased due to an increase in biomass with little further amino acid release. In contrast, amino acid productivity in the mutant strain treated with 0.1% (w/v) detergent

Fig. 1. Effects of the detergents MYRJ 45 (a, c) and MYRJ 52 (b, d) on the growth of the parent strain (a, b) and the mutant strain FT-9 (c, d). Growth was monitored by estimating the chl *a* concentration in the absence (○) and presence of the detergents at concentrations of 0.01% (w/v) (●), 0.1% (w/v) (□) and 0.5% (w/v) (■). The data shown are the means of at least three determinations.

Table 1. Photosynthetic O_2 evolution and acetylene reduction rates for cultures of mutant strain FT-9 and the parent strain when grown for 14 d in the presence of 0.1% (w/v) detergent

Each value is the mean of three replicates.

Strain	Treatment	Photosynthetic O_2 evolution [nmol h ⁻¹ (µg chl <i>a</i>) ⁻¹]	Acetylene reduction [nmol h ⁻¹ (µg chl <i>a</i>) ⁻¹]
Parent strain	Control	228	2.65
	MYRJ 45	257	1.09
	MYRJ 52	198	1.28
FT-9	Control	128	3.45
	MYRJ 45	135	1.34
	MYRJ 52	129	1.02

remained constant after reaching a maximum approximately 15 d after inoculation, implying continued amino acid liberation during growth.

The spectrum of amino acids released by the parent strain and FT-9 when grown for 14 d in 0.1% (w/v) detergent is shown in Table 2. This corresponds to the phase of growth just after the amino acid productivity per unit biomass had reached a maximum in detergent-treated mutant

Fig. 2. Effects of the detergents MYRJ 45 (*a*, *c*) and MYRJ 52 (*b*, *d*) on amino acid liberation by the parent strain (*a*, *b*) and the mutant strain FT-9 (*c*, *d*). The total amino acids in the media were estimated using a ninhydrin colorimetric assay after removal of the cells by centrifugation. Cultures were grown in the absence (○) and presence of the detergents at concentrations of 0.01% (w/v) (●), 0.1% (w/v) (□) and 0.5% (w/v) (■). The data shown are the means of at least three determinations.

strains. No amino acids were detected in the media of cultures of the parent strain in the absence of detergents but a wide range was detected in detergent-grown cultures. Both detergents induced the liberation of large amounts of unidentified ninhydrin-positive compounds. Glycine, serine, ornithine, alanine, threonine and methionine were also liberated by the parent strain when grown in the presence of either detergent. In addition, MYRJ 45 induced the liberation of valine, isoleucine and leucine, and MYRJ 52 induced the liberation of aspartate.

In cultures of FT-9 without detergent, approximately half of the total amino acids liberated into the medium consisted of alanine. This was also true in detergent-grown cultures (Table 2). However, tryptophan was not liberated in the absence of detergents but it was the second most abundant amino acid produced when FT-9 was grown in the presence of MYRJ 45. MYRJ 52 did not have this effect. Both detergents caused the liberation of small quantities of aspartate, ornithine and an unidentified component (X118).

DISCUSSION

A. variabilis was capable of growth in the presence of the polyoxyethylene stearate detergents MYRJ 45 and MYRJ 52. The data show that these detergents, when incorporated into the growth media, may increase the liberation of amino acids by a mutant strain, or induce amino

Table 2. Amino acids liberated externally by the parent strain and mutant strain FT-9 grown in batch culture for 14 d in the presence of 0.1% (w/v) detergent

Unidentified ninhydrin-positive compounds are prefixed X, followed by the retention time (min). The data presented (expressed as percentages of the total amino acids present) are from a single experimental set but are typical of several replicate experiments.

	Parent strain			FT-9		
	Control	MYRJ 45	MYRJ 52	Control	MYRJ 45	MYRJ 52
X17	—	14	18	6	6	2
Asp	—	—	2	—	1	1
Thr	—	6	3	2	2	1
Ser	—	13	5	2	3	3
Glu	—	—	—	2	4	2
Gly	—	12	11	3	4	4
Ala	—	8	3	57	42	57
Val	—	4	—	3	3	3
Met	—	—	—	1	1	1
X118	—	23	28	—	2	1
Ile	—	6	—	5	5	4
Leu	—	4	—	10	7	5
Tyr	—	—	—	2	3	1
Phe	—	—	—	5	4	4
Orn	—	4	18	—	1	1
Trp	—	—	—	—	12	—
	Totals (nmol ml ⁻¹)					
	—	20.4	9.52	50.4	43.6	60.7

acid liberation by the parent strain. MYRJ 45 can also be used to induce tryptophan liberation by the mutant strain FT-9.

The interactions between detergents and cell membranes are very complex and are dependent on many factors such as pH, detergent concentration and chemical characteristics, and membrane composition (Helenius & Simons, 1975; Tanford & Reynolds, 1976). The HLB value of a detergent is a measure of the balance between the strength of opposing lipophilic and hydrophilic groups and can be used as an empirical measure of the effectiveness of a detergent for the disruption of biological membranes, the most effective detergents having HLB values of between 12.5 and 14.5 (Tanford & Reynolds, 1976). The HLB values of MYRJ 45 and MYRJ 52 fall outside this range (11.1 and 16.9, respectively) and *A. variabilis* was capable of growth in the presence of these detergents at the concentrations employed. As the amino acids liberated in the presence of detergents were not representative of the internal free pools (Kerby *et al.*, 1987) the effects observed may therefore be due, in part at least, to specific interactions between membrane components and the detergent.

Cyanobacteria can take up and metabolize certain amino acids (Neilson & Larsson, 1980; Vaishampayan, 1982; Rawson, 1985). Energy-dependent amino acid uptake systems have been studied in *Anacystis nidulans* (Lee-Kaden & Simonis, 1982) and in *Anabaena variabilis* (Chapman & Meeks, 1983) and shown to be specific for individual amino acids or for groups of amino acids. The observed liberation of amino acids by the parent strain of *A. variabilis* and the increase in amino acid liberation by the mutant strain when grown in the presence of detergent may be due to disruption of certain amino acid transport systems as was shown for *Corynebacterium glutamicum* (Clément *et al.*, 1984; Clément & Lanéelle, 1986).

Those amino acids which were identified in the external media of detergent-grown cultures of the parent strain were mainly short-chain neutral types (glycine, alanine, serine and threonine) and ornithine and methionine. It is possible that the uptake systems which mediate the transport of these amino acids were specifically affected by the detergent. Differences between the effects of the two detergents on the spectrum of amino acids liberated may be attributed to differing specific effects of each detergent on amino acid transport systems. Those amino acids which

were detected in the media of MYRJ 45-grown cultures but not in MYRJ 52-grown cultures were all branched-chain aliphatic types (valine, leucine and isoleucine). A transport system may therefore exist in *A. variabilis* which is specific for these amino acids and which is disrupted by MYRJ 45 but not MYRJ 52.

The incorporation of detergents into the growth medium of FT-9 resulted in changes in the quantity and types of amino acids liberated. MYRJ 52 caused a substantial increase in amino acid liberation and MYRJ 45 induced the liberation of tryptophan. This amino acid is only required in small quantities in the cell and is undetectable as a free-pool amino acid by the methods employed in this study. Several factors may contribute to tryptophan overproduction, either singly or in combination: deregulation of aromatic amino acid biosynthesis at the prime branch point of the pathway, DAHP synthase; deregulation of anthranilate synthase; inhibition of the tryptophan transport system which may normally retain internally generated tryptophan; prevention of tryptophan degradation. The *in vitro* activities of anthranilate synthase of the parent and mutant strains are inhibited to a similar extent by tryptophan, whereas DAHP synthase is deregulated in the 6-fluorotryptophan-resistant strains (unpublished data). MYRJ 45 may therefore allow continued overproduction by effecting the release of tryptophan from the cell and thus preventing the inhibition of anthranilate synthase. Preliminary experiments using ^{14}C -labelled tryptophan have shown that the uptake of tryptophan by the parent strain is inhibited by MYRJ 45 but not by MYRJ 52, which supports this suggestion (unpublished data). In the absence of detergents, tryptophan degradation (Hayaishi & Stanier, 1951) may prevent the build up of the internal free pool and may account for the production of alanine by mutant strains. This possibility is currently under investigation.

We have identified three possible roles of detergents in the photoproduction of amino acids by cyanobacteria. Firstly, to increase the liberation of amino acids by mutant strains; secondly, to induce amino acid liberation by the parent strain; thirdly, to alter the range of amino acids released. The specificity with which detergents are apparently capable of affecting amino acid transport systems, and the wide range of detergents which are commercially available, may allow the development of systems which give maximum liberation of a particular product for a minimum reduction in cell viability.

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