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N<latex>$_2$</latex>-Fixing Cyanobacteria and Their Potential Applications [and Discussion]

W. D. P. Stewart, P. Rowell, N. W. Kerby, R. H. Reed, G. C. Machray, F. C. Cannon and J. Becking

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N_2-fixing cyanobacteria and their potential applications

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Cyanobacteria are N_2-fixing oxygenic phototrophs with potential as sources of nitrogenous biofertilizer independent of fossil fuels. Recently, attention has been paid to their role in this respect, and to strain selection to facilitate enhanced agricultural productivity. The capacities to fix N_2 in the presence of combined nitrogen, to resist herbicides and to tolerate salinity changes and desiccation are of particular importance. Mutant strains of N_2-fixing cyanobacteria with potential applications have been produced, including strains which photoproduce ammonia and amino acids, strains with altered transport systems and strains which are herbicide-resistant. By using bioreactors and immobilization systems in conjunction with these strains, techniques have been developed for extracellular product-liberation. Gene transfer systems are now available in both unicellular and filamentous cyanobacteria and these offer the possibility of strain selection and modification. The way in which these advances will contribute to the development of strains with desirable attributes for use in the field is discussed.

1. Introduction

Cyanobacteria are oxygenic phototrophic prokaryotes, many species of which fix N_2 via the enzyme nitrogenase (EC 1.18.2.1). Cyanobacterial nitrogenase appears to be little different from that found in other N_2-fixing prokaryotes in terms of its structural and biochemical characteristics. The *nif* gene arrangement has not been fully elucidated (see Haselkorn *et al.*, this symposium) but the indications are that the *nif* genes are dispersed, unlike their arrangement in the enteric bacterium *Klebsiella* (see Dixon *et al.*, this symposium). Whether or not a vanadium-dependent nitrogenase (see Bishop *et al.* 1980; Robson *et al.* 1986) is present in cyanobacteria has yet to be determined.

Cyanobacteria are of special interest because, in addition to fixing N_2, they also photosynthesize. This enables them to generate, in aerobic, micro-aerobic or anaerobic environments, the necessary ATP, reductant and fixed carbon essential for growth and N_2-fixation without recourse to an exogenous supply of fixed carbon. It is this dual capacity, photosynthesis and N_2-fixation within the same organism, and often within the same cell, which makes cyanobacteria attractive as sources of nitrogenous biofertilizer. Here we provide information on studies which we have carried out into the potential role of cyanobacteria as a source of biofertilizer and of other commercially important products. Progress has depended on an understanding of the basic biochemistry and molecular biology of N_2-fixing cyanobacteria.

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2. The ability of cyanobacteria to generate NH_4^+ by using sunlight, air and water

Cyanobacteria convert N_2 to NH_4^+; this is assimilated by the primary NH_4^+-assimilating enzymes glutamine synthetase (GS) (EC 6.3.1.2) and glutamate synthase (GOGAT) (EC 1.4.7.1) to generate the amino acids necessary for growth. Cyanobacterial GS is a dodecameric enzyme consisting of 12 identical subunits, each of relative molecular mass 49000–50000, as in other N_2-fixing prokaryotes (see Sampaio *et al.* 1979); however, unlike enteric bacterial GS, it is not regulated by an adenylylation–deadenylylation system (Rowell *et al.* 1977; Fisher *et al.* 1981) and its activity usually shows only a 2–3-fold difference, depending on the availability of combined nitrogen. The GS gene from *Anabaena* sp. PCC7120 has been sequenced (Tumer *et al.* 1983) and the gene is transcribed from multiple promoters, depending on the available nitrogen source. The promoter which functions under N_2-fixing conditions resembles the *Anabaena nif* gene promoters. It is possible to inhibit GS by using the glutamate analogue methionine sulphoximine (MSX) (Stewart & Rowell 1975) or hydroxylysine (Ladha *et al.* 1978). After inhibition, the NH_4^+ is liberated into the medium as shown by Stewart & Rowell (1975) for *Anabaena cylindrica*. Subsequent studies (Stewart & Rodgers 1977) showed that when symbiotic cyanobacteria are removed from their eukaryotic host they too initially liberate NH_4^+. The release of NH_4^+ appears to be due to at least three factors. First, in symbiosis the cyanobacteria show low GS and GOGAT activities (Rai *et al.* 1984). The reduction in GS activity is due, in many cases at least, to a reduction in the amount of GS protein. Second, Nierzwicki-Bauer & Haselkorn (1986) have shown that, in *Azolla*, the *glnA* mRNA level of the symbiotic cyanobacterium is reduced by about 90% compared with that of free-living *Anabaena azollae*. There is no reduction in the level of *nifH* mRNA in the symbiotic cyanobacterium although, interestingly, the ribulose-1,5-bisphosphate carboxylase small subunit message is reduced by 90%. Third, the NH_4^+ may be released in part because the NH_4^+ transport system (see Rai *et al.* 1984; Kleiner 1985), which is responsible both for NH_4^+ uptake and for the retention of endogenously generated NH_4^+, cannot retain the high endogenous pool of NH_4^+.

We have expanded on these basic findings of NH_4^+ liberation by cyanobacteria to produce a variety of bioreactors, containing entrapped cyanobacteria, capable of generating a sustained supply of NH_4^+. The bioreactors used were of the following types: packed beds, fluidized beds, parallel plates and air-lift (Musgrave *et al.* 1982, 1983*b*; Kerby *et al.* 1983). The most satisfactory, for laboratory studies, were the air-lift reactors. These contained N_2-fixing cyanobacteria (usually *Anabaena* species) which were immobilized in various gels, for example calcium alginate (Musgrave *et al.* 1982, 1983*a*, *b*) and polyurethanes (Muallem *et al.* 1983; Musgrave 1985). MSX was supplied continuously or intermittently to the bioreactors; when this was done, GS was inhibited and NH_4^+ was liberated. Such bioreactors functioned for over 800 h (see figure 1). Immobilized cyanobacteria showed little difference in morphology from free-living cyanobacteria although heterocyst frequency sometimes increased (Musgrave *et al.* 1983*a*). Long-term photosynthetic activity and nitrogenase activity were sustained in immobilized systems at rates in excess of 75% of those in free-living cyanobacteria (Musgrave *et al.* 1982). This approach demonstrated the feasibility of sustained NH_4^+ production in bioreactors with photosynthetic N_2-fixing cyanobacteria.

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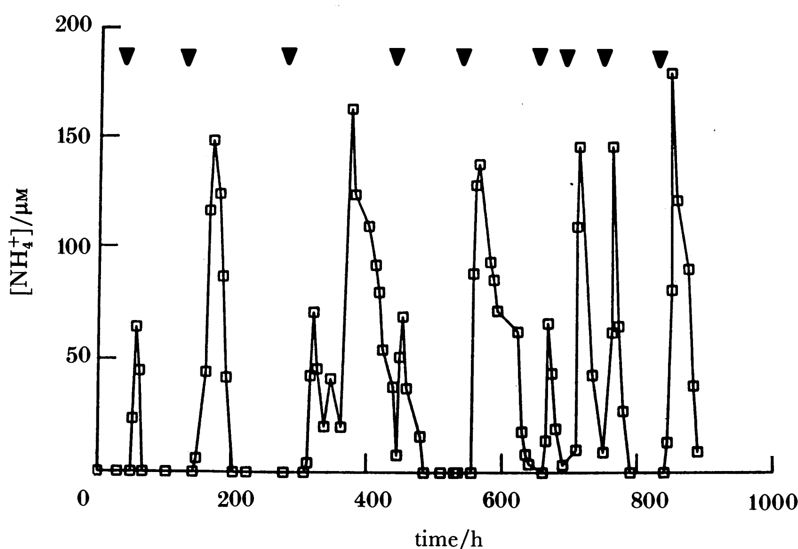

FIGURE 1. The effect of pulsing with MSX (10–50 μM, at the times arrowed) on the liberation of NH_4^+ by calcium-alginate-immobilized *Anabaena* sp. 27893 in a 750 ml air-lift reactor containing 100 g wet mass of beads (after Musgrave 1985).

3. The production of NH_4^+-liberating mutants of N_2-fixing cyanobacteria

Although the addition of MSX results in NH_4^+ release, it is a toxic compound which would have to be removed from the extracellularly produced NH_4^+ before any use. The alternative approach is to select mutants sufficiently deficient in GS activity for NH_4^+ to be released, but with sufficient GS activity to sustain metabolism. Selection for MSX resistance was used initially to generate GS-defective mutants, the assumption being that strains resistant to MSX could be deficient in GS. Although GS-defective mutants can apparently be obtained in this way (Spiller *et al.* 1986) we, like others (Chapman & Meeks 1983), have found MSX to be an unsuitable selection agent because MSX resistance can be conferred by an inability to take up MSX (transport mutants) or by GS overproduction.

An alternative and efficient approach (Polukhina *et al.* 1982; Kerby *et al.* 1985) is the selection of mutants resistant to ethylenediamine, an NH_4^+ analogue which is converted via GS to aminoethylglutamine. This product of GS is not further metabolized and accumulates. The use of ethylenediamine to generate GS-deficient mutants is based on the following findings: at pH 7.0, ethylenediamine does not enter the organism, presumably because it is not actively transported via the NH_4^+ transport system, and, at higher pH values (e.g. pH 8–9), uncharged ethylenediamine enters the cell by passive diffusion (Kerby *et al.* 1985; Rowell *et al.* 1985). Thus when ethylenediamine-resistant mutants are obtained at high pH values, they generally prove to be GS-deficient mutants and not transport mutants.

Characterization of the mutants (table 1) shows that, compared with the wild-type, they have enhanced nitrogenase activity and substantially reduced GS activity and liberate NH_4^+ (Kerby *et al.* 1986*a*). Some strains are regulatory mutants with less GS protein (e.g. strain ED92). Others, e.g. strain ED81, may be structural mutants as evidenced by their low GS biosynthetic activity but normal GS protein levels and normal GS transferase activity.

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Table 1. Growth rates, enzyme activities, protein levels and photoproduction of NH_4^+ for parent and mutant strains of *Anabaena variabilis* ATCC29413

	nitrogen source	parent strain	ED81	ED92
doubling time/h[a]	N_2	36	120	60
	ammonium (5 mmol dm^{-3})	34	120	60
	glutamine (2 mmol dm^{-3})	36	72	36
nitrogenase activity (acetylene reduction)[a]	N_2	26	68	51
	ammonium (2 mmol dm^{-3})	0	52	64
glutamine synthetase activity[b] (biosynthetic)	N_2	92	8	25
(transferase)	N_2	520	612	140
glutamine synthetase protein[b]	N_2	100	102	23
glutamate dehydrogenase activity[b]	N_2	0	0	0
alanine dehydrogenase activity[b]	N_2	40	31	46
photoproduction of NH_4^+ by immobilized strains[b]	N_2	0	44	56

All strains were grown in batch culture, under air and in the light. Enzyme activities are expressed as nmol product formed min^{-1} (mg protein)$^{-1}$. Nitrogenase activity was estimated from intact filaments, whereas all other enzyme activities were estimated from cell-free extracts. Glutamine synthetase protein levels (percentage of parental level) were estimated by immunoelectrophoresis with antiserum against *Anabaena cylindrica* CCAP 1403/2a glutamine synthetase. Growth was monitored by measuring OD at 540 nm. Photoproduction of NH_4^+ by immobilized cells is the maximum specific rate in μmol (mg Chl *a*)$^{-1}$ h^{-1}.

[a] Data from Sakhurieva *et al.* (1982).

[b] Data from Kerby *et al.* (1986*a*).

Photoproduction of NH_4^+ by such strains occurs at similar rates to those obtained by using MSX, (40–60 μmol NH_4^+ (mg Chl *a*)$^{-1}$ h^{-1}) and is sustained for over 600 h (the longest that we have tested).

GS-deficient mutants, although suitable for NH_4^+ production in the laboratory, would be unlikely to compete in the field except in certain specialized areas. A promising outlet has been the production of NH_4^+-liberating solar panels for use in irrigation channels and these are currently being tested in the field and in greenhouses.

4. The production of amino-acid-liberating mutants of N_2-fixing cyanobacteria

The production of NH_4^+ by immobilized cyanobacterial mutants provided the fundamental basis for the production of other higher-value metabolites by cyanobacteria. A range of amino-acid-liberating mutants have been obtained after NTG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine) mutagenesis and selection for resistance to the tryptophan analogue 6-fluorotryptophan and the methionine analogue ethionine (Kerby *et al.* 1987). These include broad spectrum amino acid producers (e.g. strain FT-7), essentially single amino-acid producers (e.g. strain FT-8, which liberates only alanine in quantity) and others producing families of amino acids (e.g. strain FT-2, which liberates the aromatic amino acids phenylalanine and tyrosine). The quantities produced are up to 50 mg l^{-1} in batch culture (Kerby *et al.* 1987); such

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quantities compare favourably with those obtained by using heterotrophic bacteria after initial mutagenesis and selection. By appropriate manipulation and selection, substantially increased yields should be attainable (see Atkinson & Mavituna 1983).

5. The production of other biologically active metabolites by cyanobacteria

Cyanobacteria produce a variety of biologically active compounds, including antibiotics, antifungal agents, plant growth stimulants, insecticides, pesticides and pharmaceutically important products (Lem & Glick 1985; Metting & Pyne 1986; W. D. P. Stewart, P. Rowell, N. W. Kerby & G. C. Machray, unpublished results). The great diversity and number of cyanobacterial strains and species suggests that screening will reveal additional metabolites of commercial potential.

In such studies it is important not only to have strains which produce particular compounds but, in addition, it is advantageous if such compounds are released into the medium without seriously affecting the growth of the cyanobacteria that produce them. This can be achieved in three ways. First, it is useful to select strains or mutants which naturally release the desired product. Second, it is possible to induce metabolite release by the use of detergents which increase the permeability of the plasmalemma, but which do not impair viability or growth. This technique is widely used in industry (Fukui & Ishida 1972; Clement *et al.* 1984). In tests carried out on *Anabaena variabilis* strain FT-9, which releases phenylalanine and tyrosine, but not tryptophan, tryptophan release also occurs if the detergent MYRJ-45 is added to the medium. This is probably because detergent treatment induces tryptophan

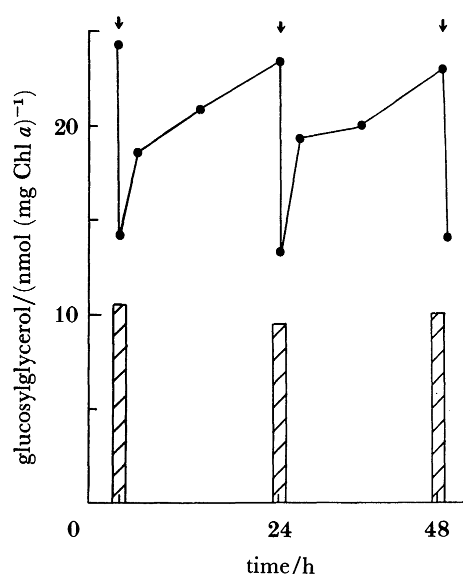

Figure 2. Changes in glucoslyglycerol concentration in immobilized cells (filled circles) of *Synechocystis* PCC6714 subjected to repeated osmotic shock (at the times arrowed). Cells grown in BGS-11 medium (Reed *et al.* 1986) were downshocked (2 min) to BG-11 medium (Rippka *et al.* 1979) at 0, 24 and 48 h and then transferred back to BGS-11 medium. The appearance of glucosylglycerol in the medium (BG-11) is also shown (hatched bars); no glucosylglycerol was detected in cells maintained in BGS-11 medium. Each value is the mean of three replicates (after Reed *et al.* (1986)).

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release, which relieves the inhibition of anthranilate synthase (EC 4.1.3.27) by tryptophan, thus allowing sustained tryptophan production (G. W. Niven, N. W. Kerby, P. Rowell & W. D. P. Stewart, unpublished data). Third, metabolites may be released extracellularly on transient salt-shock of the cells. Reed *et al.* (1986) showed that the transfer of *Synechocystis* PCC6714 and *Synechococcus* PCC6311 from high-salt medium to low-salt medium, or vice versa, resulted in a transitory (2 min) loss of plasmalemma integrity, during which time organic compounds, normally retained intracellularly, were released extracellularly. Extracellular release then ceased, presumably when membrane integrity was re-established (see figure 2). Such transitory phenomena have a negligible effect on growth and can be repeated at intervals to generate external metabolites from immobilized cells (Reed *et al.* 1986).

6. The selection and exploitation of free-living cyanobacteria for use as biofertilizer in the field

Although free-living N_2-fixing cyanobacteria have been used as sources of biofertilizer in rice fields, particularly in the Far East, since the early studies of De (1939), Watanabe (1956) and Singh (1961), the potential role of free-living cyanobacteria as sources of nitrogen input to temperate agricultural soils has only recently been established (see Jenkinson 1977).

The selection of N_2-fixing cyanobacterial strains for use in particular ecosystems is still in its infancy. By and large what has been done is to take cyanobacteria that fix N_2 well in the laboratory, grow these up in flasks or trays, dry and package them and then apply them directly to the field, or after multiplication in ponds. This technique has been adopted particularly by G. S. Venkataraman in India. Field and pot experiments, in general, suggest inputs of fixed nitrogen by cyanobacteria ranging from a few kg N ha^{-1} a^{-1} up to about 100 kg N ha^{-1} a^{-1} with an average input of 20–30 kg N ha^{-1} a^{-1} (see Fogg *et al.* 1973; Stewart *et al.* 1979; Venkataraman 1980; Roger & Kulasooriya 1980).

An aim of our current research effort is to generate N_2-fixing cyanobacteria tailored for use in particular ecosystems. Strains for use in the field should have the following features: a capacity for rapid growth on N_2; the ability to use reduced sulphur compounds, as well as water, as electron donors in photosynthesis; an efficient uptake hydrogenase to prevent wastage of nitrogenase-generated H_2; the capacity to grow heterotrophically and photoheterotrophically as well as autotrophically and the capacity to store excess fixed carbon in the light, thus sustaining nitrogenase activity in the dark; tolerance of a wide range of temperature, salinity and pH, as well as desiccation, herbicides and grazing; and the ability to continue to fix N_2 in the presence of high exogenous concentrations of combined nitrogen.

We have been examining, in particular, three such features. First, for use in the field, it is important to have N_2-fixing mutants that continue to fix N_2, unaffected by the presence of combined nitrogen. This is especially important if cyanobacteria are to be used as a supplement to chemical nitrogen fertilizer. The usual approach has been to generate mutants defective in GS activity. A major problem with this approach, as indicated above, is that mutants defective in GS grow slowly and would not compete in the field. We adopted an alternative approach of selecting for mutants which are defective in NH_4^+ transport and which therefore do not transport NH_4^+, which would inhibit nitrogenase, into the cell. To this end we have been characterizing NH_4^+ transport systems in cyanobacteria by using the analogues methylammonium ($CH_3NH_3^+$) (Rai *et al.* 1984; Rowell *et al.* 1985; Kerby *et al.* 1986*b*) and

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ethylenediamine (Kerby *et al.* 1985, 1986*a*). Studies with $CH_3NH_3^+$ show that, although there is some variation between organisms, uptake in general is biphasic with an initial phase of rapid uptake, which is energy-dependent (CCCP- and TPMP-sensitive), followed by a slower secondary phase of uptake dependent on GS activity. GS metabolizes the accumulated $CH_3NH_3^+$, resulting in sustained uptake; the product of GS (methylglutamine) is not metabolized. The use of ethylenediamine has confirmed the role of GS in sustaining NH_3 uptake, and has additionally provided evidence that nitrogenase is regulated by a product of metabolism via GS (Kerby *et al.* 1985). Selection for growth on toxic levels of $CH_3NH_3^+$ has resulted in the generation of a variety of $CH_3NH_3^+$-resistant mutants. In some cases, e.g. *Anabaena variabilis* strain 4m3, the uptake and subsequent metabolism of both NH_4^+ and $CH_3NH_3^+$ are reduced and nitrogenase activity becomes markedly less affected by exogenous NH_4^+ (figure 3). GS activity of strain 4m3 is similar to that of the parent strain (A. Reglinski, N. W. Kerby, P. Rowell & W. D. P. Stewart, unpublished results). Such NH_4^+-transport mutants are important not only because they grow normally, and show sustained nitrogenase activity even in the presence of combined nitrogen, but also because, combined with the gene-transfer systems that are now available for cyanobacteria (see below), they may prove to be valuable for the isolation of the genes involved in NH_4^+ transport in cyanobacteria.

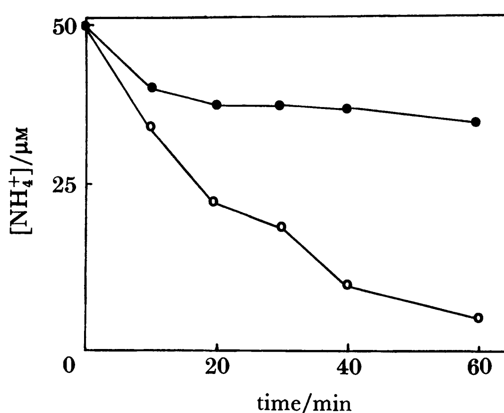

FIGURE 3. The uptake of NH_4^+ at pH 7 by a wild-type (open circles) and a methylammonium-resistant strain (4m3) (filled circles) of *Anabaena variabilis* ATCC 29413 (A. Reglinski, N. W. Kerby, P. Rowell & W. D. P. Stewart, unpublished).

Second, it is important that N_2-fixing cyanobacterial strains for use in the field are resistant to commonly used herbicides. This is especially important for herbicides which affect higher-plant photosynthetic electron transport and amino acid biosynthesis because these will have a similar effect on cyanobacterial photosynthesis and metabolism and will thus kill off the cyanobacterial flora in the soil, thereby reducing the biological nitrogen input. Additionally, cyanobacteria are particularly useful for genetical studies on herbicide resistance because they enable techniques of microbial genetics to be readily applied to photosynthetic and, in some cases, biosynthetic systems similar to those of higher plants. Cyanobacterial mutants resistant to the photosynthetic electron-transfer inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (see, for example, Golden & Sherman 1984; Golden & Haselkorn 1985) and glyphosate (N. W. Kerby, P. Rowell & W. D. P. Stewart, unpublished results) are available

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and; the gene for acetolactate synthase (EC 4.1.3.18), the target enzyme for the sulphonylurea and imidazolimine herbicides, has been cloned from *Anabaena* (Mazur *et al.* 1985).

Third, particular attention has been paid to the question of how cyanobacteria achieve tolerance of salinity and desiccation. Studies in our laboratory (Reed *et al.* 1984*a*, *b*, 1985*a–c*; Reed & Stewart 1985) and elsewhere (Mackay *et al.* 1983, 1984; Borowitzka & Brown 1984) indicate that cyanobacteria overcome high salinity, and to some extent drought, by accumulating one of several major organic osmotica, some of which are unique to cyanobacteria, for example, glucosylglycerol and glutamate betaine (Reed & Stewart 1983, 1985; Reed *et al.* 1984*a*, *b*, 1985*b*). These osmotica also serve as compatible solutes, protecting enzyme activity from salt-inactivation in osmotically stressed cells (Warr *et al.* 1984) and replacing inorganic ions as major components of cell turgor during long-term growth in saline media (Reed *et al.* 1985*a*, *c*). Figure 4 provides evidence that some cyanobacteria, at least, may

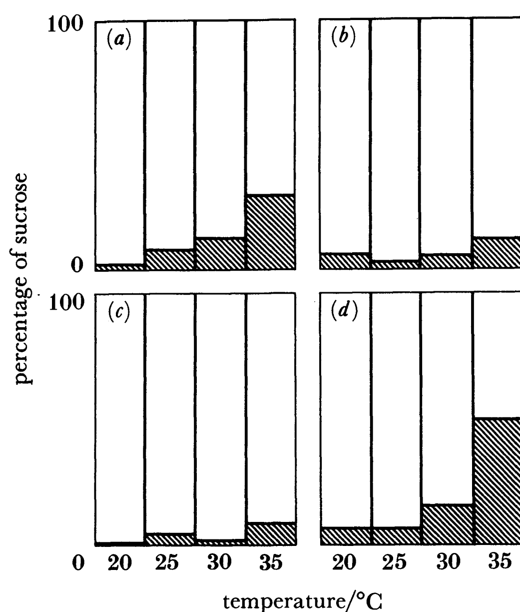

FIGURE 4. Proportions of glucosylglycerol (open areas) and sucrose (hatched) in four isolates of *Synechocystis* grown in 100% seawater medium for 15 d at temperatures from 20 to 35 °C. (*a*), strain PCC6714; (*b*), strain PCC7008; (*c*), strain PCC6806; (*d*), strain PCC6702. (From Warr *et al.* (1985*b*).)

switch from the production of one organic compound to another with changing environmental conditions such as temperature (Warr *et al.* 1985*a*, *b*). By using such basic biochemical information for cyanobacteria, together with recently developed techniques of genetic engineering, it is now realistically possible to obtain N_2-fixing cyanobacterial strains for use in particular ecosystems and soil types.

7. Gene transfer systems for cyanobacteria

Until recently there were no well-developed, readily reproducible gene transfer systems for cyanobacteria, unlike (for example) the enteric N_2-fixing bacteria such as *Klebsiella* (for reviews, see Dixon 1984; Dixon *et al.*, this symposium) where progress benefited greatly from

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Table 2. Representative transformation systems for unicellular cyanobacteria

cyanobacterial DNA and/or plasmid	*E. coli* DNA and/or plasmid	vector	antibiotic resistance	host range	transformation efficiency/µg DNA	reference
Anacystis nidulans DNA	—	—	—	*A. nidulans*	1.5×10^{3}	Shestakov & Khyen (1970)
A. nidulans: pUH24	Tn 901	pCH1–5 pUC1	Ap^{R}	*A. nidulans*	1.5×10^{3}	Van den Hondel *et al.* (1980)
A. nidulans: hybrid plasmid pUC1	pACYC184	pUC104–105	Ap^{R} Cm^{R}	*A. nidulans* *E. coli*	1.5×10^{5} 5.0×10^{5}	Kuhlemeier *et al.* (1981)
A. nidulans: pBA1	pBR322	pBAS18	Ap^{R}	*E. coli*	—	Shinozaki *et al.* (1982)
A. nidulans: pUH24	pBR322::Cm	pLS103	Ap^{R}	*A. nidulans* *E. coli*	1.0×10^{5} 7.0×10^{5}	Sherman & Van de Putte (1982)
Agmenellum quadruplicatum pAQ1	pBR322	pAQE2	Ap^{R}	—	—	Buzby *et al.* (1985)
		pAQE12	Ap^{R}	*Agmenellum* *E. coli*	4.6×10^{4} —	
pAQE12	50bp:PUC7	pAQE15	Ap^{R}	*Agmenellum* *E. coli*	— —	
pAQE15	pRZ102	pAQE17	Ap^{R} Km^{R}	*Agmenellum* *E. coli*	1.3×10^{5} —	
A. nidulans: pUH24	pBR325	pECAN1	Ap^{R} Cm^{R}	*A. nidulans* *E. coli*	5.0×10^{2} —	Gendel *et al.* (1983)
A. nidulans: pUH24	pDPL13	pPLANB2	Ap^{R}	*A. nidulans* *E. coli*	8.4×10^{3} —	
A. nidulans: pUC1	Sm^{R} from pRI477S	pUC12	Ap^{R} Sm^{R}	*A. nidulans*	1.0×10^{6}	Kuhlemeier *et al.* (1983)
pUC12–Ap^{R}		pUC13–14	Sm^{R}	*A. nidulans*	2.7×10^{6}	
pUC13	pACYC184	pUC303	Sm^{R} Cm^{R}	*A. nidulans* *E. coli*	1.8×10^{6}	
A. nidulans: pUH24	pBR325	pDF30	Ap^{R} Cm^{R}	*A. nidulans* *E. coli*	2.0×10^{3} 5.0×10^{4}	Friedberg & Seijffers (1983)
	pBR322	—	Ap^{R} Tc^{R}	*A. nidulans* *A. nidulans* permeaplasts	8.0×10^{5} 4.0×10^{6}	Daniell *et al.* (1986)
Synechocystis 6803: pUS2, pUS3	pACYC177	pUF12	Km^{R}	*Synechocystis* 6803 *E. coli*	30 —	Chauvat *et al.* (1986)
		pUF3	Km^{R}	*Synechocystis* 6803 *E. coli*	30 — —	
pUF3 + pUS2, pUS3		pUF311	Km^{R}	*Synechocystis* 6803 *E. coli*	— —	
pUF311	Cm^{R} from pACYC184	pFCLV7	Km^{R} Cm^{R}	*Synechocystis* 6803 *E. coli*	2.0×10^{3} —	
Synechocystis 6803: pUS2, pUS3	pACYC184	pSE7	Cm^{R}	*Synechocystis* 6803	—	S. V. Shestakov (unpublished)
pSE7 + pUS2		pSE76	Cm^{R}	*Synechocystis* 6803	1.0×10^{4}	
pSE76	pUC-4K	pSE176	Km^{R} Cm^{R}	*Synechocystis* 6803 *E. coli*	— —	

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the information which was available for the related *Escherichia coli*. Now a variety of gene transfer systems for unicellular cyanobacteria have been described, the organisms most frequently used being strains of *Anacystis nidulans*, *Agmenellum quadruplicatum* and *Synechocystis* (see table 2). The most satisfactory gene transfer systems are the hybrid shuttle vectors, which consist of hybrid *E. coli* – cyanobacterial plasmids, from which, in some cases, cyanobacterial restriction-enzyme sites have been deleted and where incorporation of cyanobacterial DNA either affords stability to the replication of the hybrid plasmid in the cyanobacterial host or facilitates integration into the chromosome.

Recently, Wolk *et al.* (1984) developed a triparental method of mating for filamentous cyanobacteria; this involves the use of two parental *E. coli* strains, one bearing the broad-host-range plasmid RP4 and the other the hybrid shuttle vector which is to be transferred to the recipient cyanobacterium, plus a helper plasmid to aid in its transfer. This method, although effective, suffers from a disadvantage in that it is difficult to routinely free the exconjugant cyanobacteria from contamination with *E. coli* after mating (table 3). We have developed a modified and improved system in our laboratory, in which a single parental *E. coli* carries both the shuttle vector and all the functions necessary for its transfer (table 3). The parental *E. coli*

TABLE 3. CONJUGATION SYSTEMS FOR GENE TRANSFER IN HETEROCYSTOUS CYANOBACTERIA

type of conjugation	donor strains	recipient strain	plasmid	antibiotic resistance	reference
triparental	*E.coli* HB101+J53 (RP-4)	*Anabaena* sp. PCC7120 *Anabaena* sp. PCC7118 *Anabaena* sp. U.Tokyo M-131 *Nostoc* sp. PCC7524 *Nostoc* spp. ATCC 27895, 29133, 29150	pRL series	Cm^R Km^R Sm^R Em^R	Wolk *et al.* (1984) Flores & Wolk (1985)
biparental	*E. coli* S 17-1	*Anabaena* sp. ATCC27893	pRL series	Km^R Cm^R Sm^R Em^R	McFarlane *et al.* (1987)

strain S17-1, contains RP4-2-Tc::Mu-Km::Tn7 integrated into its chromosomal DNA. This strain also lacks an *E. coli* K-12-specific DNA restriction system thus allowing efficient uptake of foreign cloned DNA. pDS4101 (colK::Tn1), a helper plasmid which supplies mobility function *in trans* has been introduced by transformation into this host followed by a shuttle vector (either pRL10 or pRL6 with selection for Cm^R). The filter conjugation method was carried out as described by Wolk *et al.* (1984). Axenic cyanobacterial exconjugants thought to have received the shuttle vector were identified by their antibiotic resistance characteristics, the presence of the shuttle plasmid and its identical restriction map to the shuttle plasmid of the donor (McFarlane *et al.* 1987). The advantages of the biparental system are that all *E. coli* cells are potential donors and that the method allows counterselection against donor *E. coli* by using kanamycin, to which *Anabaena* sp. ATCC27893 is naturally resistant. The system works particularly well with pRL6 and pRL10. With pRL1, evidence of recombination of the hybrid shuttle vector with an endogenous resident cyanobacterial plasmid has been observed, as Flores & Wolk (1985) have noted for the triparental conjugation and Kuhlemeier *et al.* (1985) have noted with unicellular cyanobacteria. Such gene-transfer systems, coupled with the use of various mutants, offer the possibility not only of strain selection and strain tailoring for

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cyanobacteria, but also provide a mechanism for the elucidation and characterization of the function of various cyanobacterial genes.

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Discussion

F. C. CANNON (*Biotechnica International Inc., Cambridge, Massachusetts, U.S.A.*). Regarding the need to perfuse the immobilized cyanobacteria with inhibitors in order to obtain excretion of ammonia, have Professor Stewart and colleagues tried using mutants defective in glutamine synthetase?

W. D. P. STEWART. Yes.

J. BECKING (*Research Institute ITAL, Wageningen, The Netherlands*). I am interested in the basis for Professor Stewart's conclusion that free-living cyanobacteria yield more fixed nitrogen than

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symbiotic forms, such as *Azolla* spp., for the growth of the rice plant. Because of the wide range of responses of mineralization of organic nitrogen to environmental conditions, it is necessary to specify the test conditions in which such comparisons are made. In our experiments with ^{15}N-labelled *Azolla*-N and monitoring its uptake in the rice plant in various soils differing in structure, N-content, pH, water content (normal and waterlogged), etc., we found, at least for some soils, a more positive result.

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