N91-31786

CYANOBACTERIA IN CELSS: GROWTH STRATEGIES FOR NUTRITIONAL VARIATION AND NITROGEN CYCLING

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ABSTRACT.

Cyanobacteria (Blue-Green Algae) are versatile organisms which are capable of adjusting their cellular levels of carbohydrate, protein and lipid in response to changes in the environment. Under "stress" conditions (salinity, cold shock) there is an imbalance between nitrogen metabolism and carbohydrate/lipid synthesis. The lesion in nitrogen assimilation is at the level of transport: the "stress" condition diverts energy (trans-membrane pH gradient) from the active accumulation of nitrate to the extrusion of salt; and probably inhibits a cold-labile ATP'ase in the case of cold-shock. Both situations affect the bioenergetic status of the cell such that the nitrogenous precursors for protein synthesis are depleted. Despite the inhibition of protein synthesis and growth, photosynthetic reductant generation is relatively unaffected. The high O_2 and reductant would normally lead to photo-oxidative damage of cellular components, however the organism copes by channeling the "excess" reductant into carbon storage products. The increase in glycogen (28-35% dry weight increase) and the elongation of lipid fatty acid side chains (2-5% dry weight increase) at the expense of protein synthesis (25-34% dry weight decrease) results in carbohydrate, lipid and protein ratios that are closer to those required in the human diet. In addition, the selection of nitrogen fixing mutants which excrete ammonium ions present an opportunity to "tailor" these micro-organisms to meet the specific need for a sub-system to reverse potential loss of fixed nitrogen material.

INTRODUCTION.

To date, life support systems in manned space flights have consisted of consumables transported in a spacecraft for the duration of the flight and periodically replenished by subsequent space flights during longer missions such as Skylab. This has worked well on short missions. However, a contained self-regenerating system (1) that produces edible biomass (1,2) from crew waste products and sunlight would have clear advantages and has been proposed by the Controlled Ecological Life Support System (CELSS; 2). The proposed CELSS is an integration of several unifunctional, tightly

controlled sub-systems such that the output of one sub-system provides the required input of the next sub-system in the cycle. The major sub-systems are shown in figure 1. However, in this "closed-cycle" not all components are inherently stable but must be controlled or regenerated. One obstacle to maintaining system stability is posed by the loss of fixed nitrogen during waste processing and biological denitrification.

Photosynthetic organisms are of prime consideration (1) in a CELSS since they are capable of producing biomass from simple inorganic compounds at the expense of light energy by the so-called oxygenic photosynthesis. Since these photosynthetic systems generate O_2 (from the photolysis of H_2O), they could augment the physical/chemical air revitalization subsystems.

One group of photosynthetic organisms under consideration are the highly versatile blue green algae (3, 4). These microscopic blue green algae blend the advantages of higher plants (photosynthesis) with the ease of handling of bacteria (axenic cultures grown in fermentors). These organisms produce a high percentage of their biomass as protein (table 1), and we have demonstrated that the metabolic energy produced by photosynthesis can be redirected to carbohydrate and lipid synthesis and away from protein, by simple manipulations of environmental factors. With these methods, we are able to manipulate the productivity of protein, lipid, and carbohydrate in a single organism to levels which are compatible with the human dietary requirements.

One distinct advantage of some species of blue green algae is their ability, under nitrogen limitation, to reduce atmospheric N_2 to a biologically useful form. This would provide the CELSS with a sub-system for maintaining the nitrogen balance, countering potential losses due to

denitrifying bacteria or physical processing, projected in the waste processing subsystems (5, 6).

CARBON-NITROGEN BALANCE AND CONTROL.

A basic concept of photosynthesis, outlined in Figure 2, is the competition between N_2/NO_3^- and CO_2 for reductant and ATP. That is, of course, a rather simplistic picture, since CO_2 reduction is required to provide the carbon skeleton for assimilation of NH_3 into amino acids (proteins), and nitrogenous material is incorporated into some lipids, nucleic acids and cell-wall components. Generally, however, the competition between CO_2 and nitrogen reduction does hold true; and since photosynthetic electron flux (and hence reductant supply) is not shown to be improved by environmental factors, the theoretical productivity of one component of the biomass (e.g. carbohydrates) could only be increased at the expense of another (e.g. protein). Our results show this to be the case. Using "shock" (i.e. salinity or cold treatment), we have been able to demonstrate the partitioning of reductant away from (excess) protein to (required) carbohydrate and lipid. Growth of the unicellular Synechococcus 6311 as measured by increments in chlorophyll, was inhibited ~30% with 0.5M NaCl (figure 3a), and since the protein content per cell also decreased, this demonstrates an approximate 50% reduction in total protein productivity. Although the growth rate is inhibited, the glycogen productivity increases markedly, by a factor of 10 over control cells (Figure 3b). In addition, the lipid content was also found to change. Not only did the total lipid increase by 5%, but the fatty acid composition was also altered. Under salt shock the length of the fatty acids were increased from 16:1 to 18:1. Analysis of the total caloric content of the carbohydrate (glycogen plus soluble sugars), protein and lipid per gram of cells under "stress" is shown in table II. The

total caloric value per gram of cells remains fairly constant, but the source of the calories changes, this redirection of reductant truly represents a shift of metabolism from protein to carbohydrate synthesis.

The mechanism involved in this shift in metabolism seems to involve the energy status of the cell. We have extensively documented the physiological and biochemical response of Synechococcus 6311 to salt (7-14), and recent results point conclusively to a depolarization of the trans cytoplasmic membrane pH gradient as a primary event. We have shown that the membrane pH gradient, generated by a membrane bound ATP'ase under non-stress conditions and directly by respiration under stress conditions, is responsible for driving the accumulation of nitrate into the cell. This pH gradient is also utilized to remove excess intracellular sodium ions via a Na^+/H^+ antiporter (13). The presence of high concentrations of intracellular salt compete with nitrate for the pH gradient (table III), with the net result that the cell's uptake of nitrate is severely inhibited and photosynthetic reductant is channeled into CO_2 fixation. The mechanism involved in the case of cold shock is less clear, but there are indications that the cold-labile ATP'ase may be partially inhibited, which would deplete the magnitude of the pH gradient and hence inhibit the uptake of nitrate.

In summary, the utilization of energy (ATP) for non-growth functions (salt removal) or energy depletion (ATP'ase inhibition) results in the inhibition of the nitrate uptake mechanism, presenting a nitrogen starvation situation which results in "over-production" of fixed carbon compounds. This scenario may be capitalized upon in future research, for example, one can envisage the selection of suitable mutants defective in their ATP'ase function, which would synthesize sufficient carbohydrate for human dietary

requirements without the need for environmental manipulations.

Selection of mutants as a way to "tailor" micro-organisms to meet specific needs of sub-systems within the proposed CELSS presents us with a powerful tool. In the next section we will present an overview of how a specific mutant of nitrogen-fixing cyanobacteria may be used as one component in the CELSS.

NITROGEN CYCLING

Organic (fixed) N_2 could be lost from a CELSS due to denitrifying bacteria in stored waste material or by oxidation to N_2 in the proposed waste management system such as catalytic wet oxidation (CWO; 5) and/or supercritical water oxidation (SCWO; 6). Current physical/chemical systems for the reduction or oxidation of N_2 to NH_4^+ or NO_3^-/NO_2^- have a high energy expenditure (approximately 20,000 KWh per ton of nitrogen fixed) in the case of the Haber process, a low efficiency (2%) in the case of the Birkland-Eyde electric arc process, or system instability in the case of metal complexes as catalysts (for a recent review on man-made N_2-fixing systems see 15). Photosynthetic nitrogen fixation by cyanobacteria is a plausible means of generating a pool of biologically usable fixed nitrogen. Cyanobacteria are a rich source of nitrogen, the majority of which is in a biologically useful form, protein (table IV). However, the mode of transfer of the fixed nitrogen back into the cycle is a critical consideration. The simplest method would be to use the cyanobacteria as a protein supplement for the crew, who become the "nitrogen processing sub-system" (figure 1). An attractive alternative is to select a mutant with the ability to excrete a nitrogenous product that can be used directly by the plant growth chamber. There are several reports in the literature in which versatile cyanobacterial strains have been used with the

specific aim of excreting NH_4^+ ions (produced by nitrogen fixation) into the medium (16-20), and NH_4^+ ions are an ideal nitrogen source for hydroponics. The filamentous cyanobacteria can convert atmospheric N_2 (as a sole nitrogen source; 16, 21, 22) into NH_4^+ using only minerals, CO_2 and light (see ref. 8 and 13 for relevant reviews of N_2 fixation). In these bacteria photosynthesis occurs simultaneously with the oxygen sensitive nitrogen fixation process. To achieve this state, the nitrogen fixing apparatus (nitrogenase) is housed in a specialized, differentiated cell (the heterocyst; 23) where the partial pressure of O_2 is maintained at a low level (24). This unique arrangement allows the cyanobacteria to photo-produce NH_4^+ ions and carbohydates and, therefore grow on a minimal medium without the need for added complex carbon compounds. Under conditions where alternative fixed nitrogen sources (NH_4^+, NO_3^-) are available to the cell, the nitrogenase complex is not expressed and heterocyst differentiation is inhibited (23, 26).

The second key enzyme in the metabolic pathway of nitrogen is glutamine synthetase (GS). Glutamine synthetase is expressed irrespective of the source of nitrogenous material, and is the first step in the incorporation of NH_4^+ ions into amino acids. This enzyme removes NH_4^+ ions from the cytoplasm, combining then with glutamate to produce glutamine (25-28) which can be incorporated into protein or transaminated by glutamine 2-oxoglutarate amino transferase (GOGAT; 16) to regenerate the glutamate and synthesize the required amino acids from carbon precursors. To achieve a build-up of NH_4^+ ion concentration, it is necessary to inhibit GS activity. One of the major techniques which has been used to induce strains of nitrogen fixing bacteria to excrete NH_4^+ ions, is the GS inhibitor methionine sulfoximine (MSX; 17, 18). However, MSX is extremely toxic and its removal from the recycled

nutrients would prove problematic. An alternative technique is the production and selection of mutants that excrete high levels of NH_4^+ ions. The use of NH_4^+ analogs such as ethylenediamine or methylamine has been used successfully to select mutants deficient in GS activity, while retaining high nitrogen fixing capabilities (16, 19, 20). The technique is based on exposure of cells (previously grown on nitrogen deficient media) to a mutagen (nitrosoguanidine; 19). The mutated cells are then exposed to an NH_4^+ analog (ethylenediamine) at pH 9 which allows the ethylenediamine to passively diffuse into the cell and prevents selection of NH_4^+ transport mutants (20). Because ethylenediamine is metabolized by GS to produce aminoethylglutamine, a compound which is not metabolized further and accumulates, mutants with a low GS activity accumulate aminoethylglutamine more slowly. Thus, the survival rate of the cells is higher, presumably because less of their glutamate is "tied-up" as aminoethylglutamate and they can assimilate NH_4^+ ions produced by the nitrogenase complex.

A second effect is that the accumulation of aminoethylglutamate causes inhibition of nitrogenase activity (20). In mutants with the nif genes derepressed, accumulation of the aminoethylglutamine will have little effect, allowing N_2 reduction to continue. The net result of this procedure is to select for a double mutant. In such a system (in the absence of NH_4^+ analogs) there is an imbalance between the rate of NH_4^+ supplied by the nitrogenase complex and the rate of assimilation into amino acids. This results in a release of NH_4^+ ions into the medium (see figure 4), and the longevity of these mutants is promising (up to 600 hr. tested so far; 16).

Stewart et al have considered the use of such mutants to supply nitrogen directly to crops in the field, but have concluded that competition from the faster growing wild type cyanobacteria would probably reduce their

effectiveness (16). However, such competition would not exist in a controlled bioreactor, making this an ideal system for inclusion as part of a CELSS.

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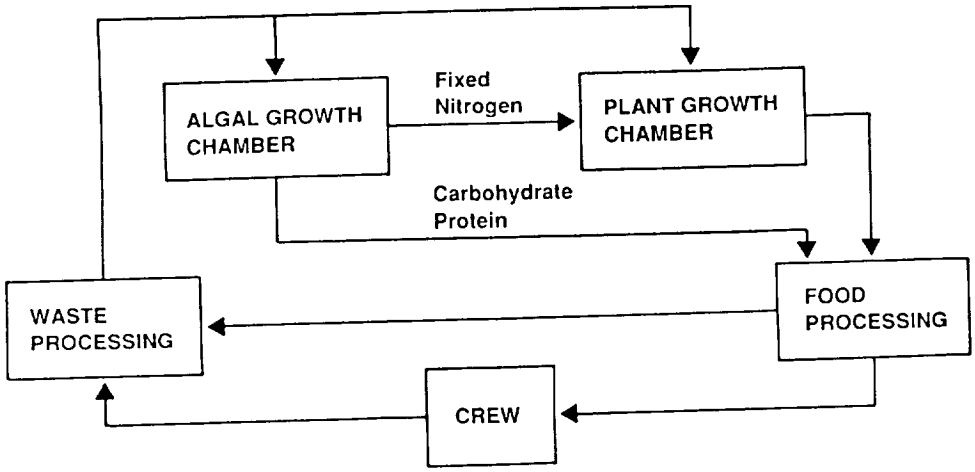

Figure 1. Major components within a CELSS.

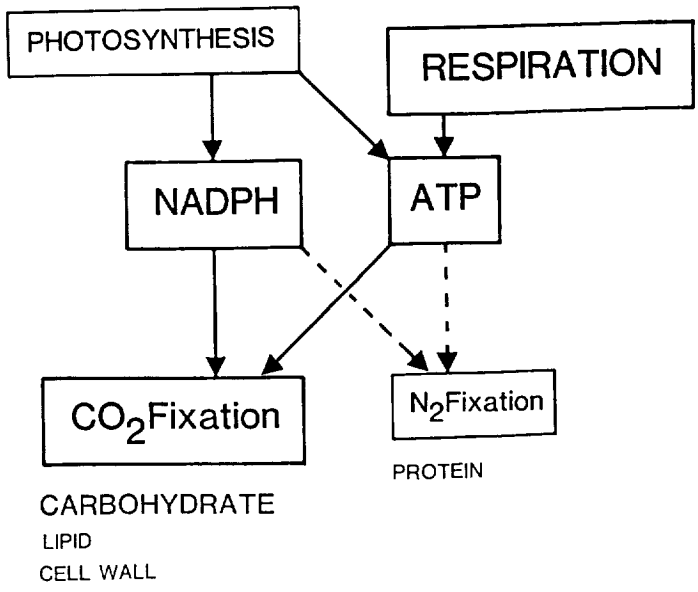

Figure 2. Energy flow diagram, competition between CO_2 and N_2 for reductant and ATP.

Table I. Storage granules in cyanobacteria.

Storage granule (Cell inclusion)	% dry weight	Present in Synechococcus	Present in Spirulina
GLYCOGEN	5-60	yes	yes
POLY-B-HYDROXY-BUTYRATE (lipid)	6(a)	n.a.	yes
MEMBRANES	12-16 (b)	yes	yes
CARBOXYSOMES (protein)	25	yes	yes
PHYCOBILSOMES (protein)	10-20 (a,b)	yes	yes
CYANOPHYCIN (protein)	8-12 (a,c)	no	yes?
GAS VESICLES (protein)	2 (d)	no	yes

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Table II.

Cellular composition and energy content of Synechococcus 6311.

	Control		Salt shock		Cold shock	
	%DWt	Kcal/g cells	%DWt	Kcal/g cells	%DWt	Kcal/g cells
CARBOHYDRATE	6.0	0.24	29.2	1.17	38.2	1.53
PROTEIN	67.0	2.68	43.6	1.74	33.2	1.33
LIPID	15.0	1.35	15.2	1.37	16.6	1.49
		4.27		4.28		4.35

Energy content (Kcal/g DWt cells) calculated assuming conversion factors of 4, 4 and 9 Kcal/g for carbohydrate, protein and lipid respectivly (Bugbee, B. G. and Salisbury, F. B. in; Controlled Ecological Life Support Systems: CELSS'85 Workshop (MacElroy, Martello and Smernoff eds.) pp447-486, 1986)

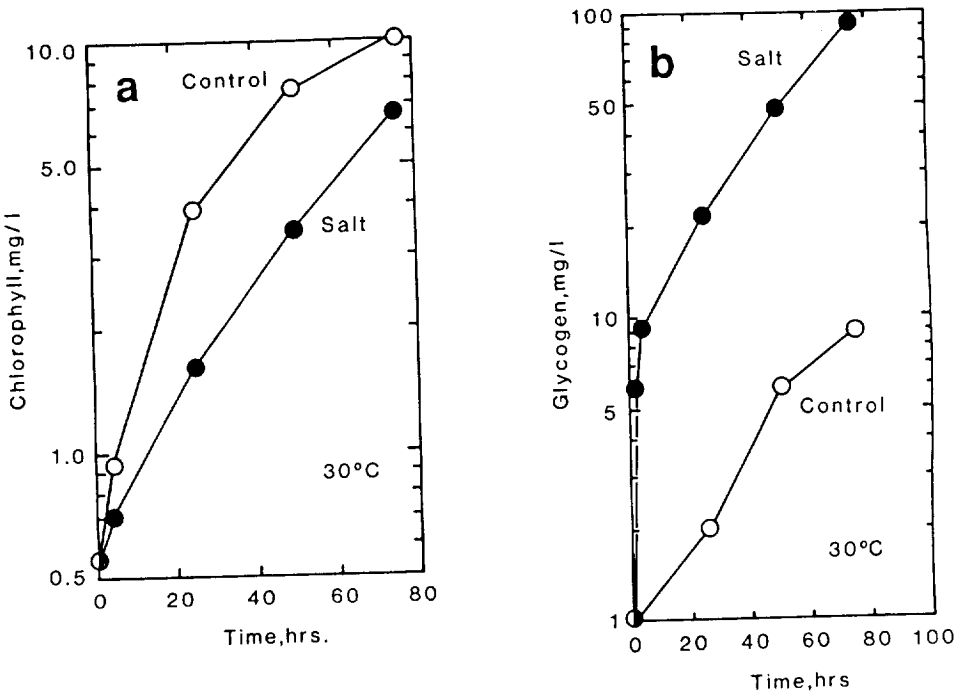

Figure 3. Effect of salt (0.5M NaCl) on the growth (a) and glycogen productivity (b) in Synechococcus 6311.

Table III.

Rate of nitrate uptake by Synechococcus 6311.

Additions	NO_3^- uptake, umoles/mg chlorophyll/hr.
none	0.75
NaCl, 10 mM	0.57
NaCl, 100 mM	0.12

with an imposed pH gradient of 2 units (acid outside) and in the presence of KCN (2 mM) and DCCD (2 umoles/mg chlorophyll).

Table IV.

Nitrogen in Cyanobacteria

Protein	60-70 % Dry Weight
Nitrogen	87 % as Protein
	13 % as Nucleic Acid and Peptidoglycan

8-9g N per 100g cells (Dry Weight)

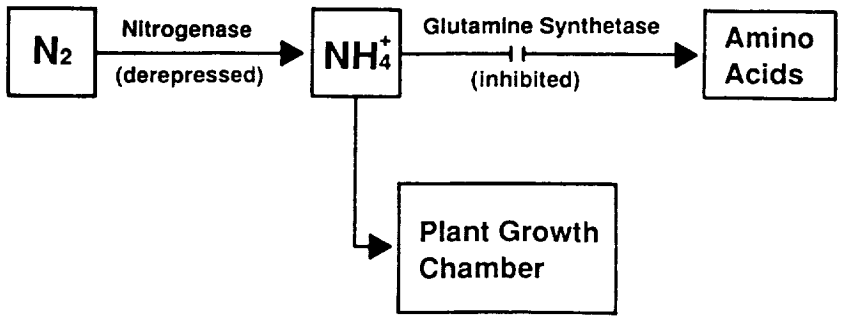

Figure 4. Nitrogen flow diagram in the proposed mutant of a nitrogen-fixing cyanobacterium.