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Studies on the molecular biology of the cyanobacteria *Spirulina maxima*

Lee, Clark Philip, Ph.D.

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STUDIES ON THE MOLECULAR BIOLOGY OF THE CYANOBACTERIA *SPIRULINA MAXIMA*

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN BIOMEDICAL SCIENCES (BIOCHEMISTRY)

DECEMBER 1989

by

Clark P. Lee

Dissertation Committee:

Morton Mandel, Chairman
N. V. Bhagavan
Howard Mower
Suresh Patil
John Stiles

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To those who have tried and failed.

BOBO

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ABSTRACT

Early in 1983, we began a study on the cyanobacterium *Spirulina sp.*, stemming from a growing interest in the commercial production of this algae in the Hawaiian islands. The algae is grown primarily as a food supplement due to it's exceptional protein quality. It was proposed that through genetic manipulation, one might be able to further enhance the commercial exploitation of this algae. Although the molecular biology and techniques of genetic engineering in cyanobacteria has advanced rapidly in the past few years, the application to *Spirulina* has been minimal. The objective of this work was therefor to; 1) generate a shuttle vector and method of mobilization and 2) clone and characterize the phycobiliprotein genes for the purpose of exploiting these high expression, light regulated genes.

To start, 15 available strains were screened for endogenous plasmids using the methods in the literature as they developed. The uniform response of the test strains to heavy metal and antibiotic tolerance (specifically to chromium and kanamycine), encouraged the pursuit of all avenues of detection. Unable to detect any plasmids in any strain, an attempt to clone a labile or megaplasmid (or origin of replication) into the E.coli replicating pACYC184 or the *Anacystis nidulans* shuttle vector pPUC29 (Van

denHondel et.al.) was investigated. With this "shotgun" library experiments were established to transform the algae and select for the potent chloramphenicol marker.

Transfomation conditions for cyanobacteria have generated numerous publications and we attempted to repeat most of them using the above mentioned vectors and libraries. The only conditions tested showing any increased survivability was when the algae was grown in iron depleted media and selecting by underagar injection. However, no cultures survived reselection.

It became apparent that the presence of a restriction system required investigation. Whitehead et.al. has published a procedure for the rapid screening for type II restriction enzymes. Using this method, results show an extremely powerful DNAse is present in *Spirulina*. Wether this is a restriction system or a nonspecific DNAse is not clear in that we were unable to determine a set of conditions that would produce digestion patterns other than a smear of low molecular weight DNA. This would apparently limit transformation as a method for gene transfer. The alternitive method of conjugation has been attempted, however the plating of the mating mixture on mixed media (Spirulina media + 0.5% LB) resulted in mixotrophic lysis of the algae.

Finally, in regard to the cloning of the phycobiliprotein genes, some unusual problems presented

themselves. Using the clone pTP1,and fragments thereof, from *Agmenellum quad.* (Pilot) as probe DNA, dozens of hybridization conditions were attempted. Only under the conditions of Lemeaux and Grossman (ref.)were we able to obtain definitive signals and only then when the genomic DNA was digested with EcoR1, BglII, ClaI and PstI . The explanation for this is not evident from the data obtained in that the base composition, degree of methylation, and C-PC N-terminal amino acid sequence data are not unusual. The stringency conditions required, based on salt and temperature are not unique other than the absence of DNA and protein blocking agents. Further, colony hybridization for library screening was unsuccessful. Only through hybridization of Southern blots of minipreps run on a gel were we able to isolate four unique clones.

The clones obtained are from sized Eco/Bgl digests of *S.maxima* genomic DNA cloned into pBR322 and screened using the alpha structural region of pTP1 as a probe. The restriction patterns and preliminary sequence data for the EcoRI and BglII ends are unique to each clone. They are similar in that most sites useful for subcloning into polylinker regions were not detected. Digesting with BstYI(XhoII) gave identical fragments, common to the alpha probe, for every clone however subcloning of these fragments has so far been unsuccessful.

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LIST OF ABBREVIATIONS

AMP = ampicillin
APC = allophycocyanin
ATCC = American Tissue and Culture collection
CAM = chloramphenicol
C-PC = C-phycocyanin
DD H_2O = double distilled water
DNA = deoxyribonucleic acid
EDTA = ethylene diamine tetraacetic acid
het = heterocyst forming gene
KAN = kanamycine
nif = nitrogenase gene
ORF = open reading frame
PCC = Pasture Culture Collection
PE = phycoerythrin
RUBPCase or RuBISCo = ribulose-bis-phosphate carboxylase -oxygenase
SSC = sodium chloride, sodium citrate buffer
SSPE = sodium chloride, sodium phosphate, EDTA buffer
TET = tetracycline
UTEX #### = University of Texas at Austin algal collection identification number

PREFACE

The cyanobacteria Spirulina sp. is best known as a health food used as a protein and B-vitamin supplement. It has long been in use in western cultures and is of historical significance in the regions of Lake Chad, Africa and Lake Texcoco, Mexico where the locals survived on the algae in times of famine and as an alimentary supplement. The historical and anthropological significance of spirulina in the Lake Texcoco area of Mexico, referred to as "tecuitlatl" (the stones excrement), is documented by Farrar. Dr. Farrar has accumulated documents and letters of the Spanish conquest of the Aztec city, Tenochtitlan, on Lake Texcoco in Mexico. Letters from Diaz, Cortez and several friars document the consumption of a slime collected from the lake with nets, dried and sold in the market. The slime was described as a clear blue mud that breeds on the water. Although impossible to verify, the slime was probably Spirulina.

The natural, virtually pure culture that exists in Lake Texcoco, is so abundant that it has been harvested in the hundreds of tons per year by the neighboring soda ash plant and until recently was the worlds leading supplier of the algae. However, the mining operations in the lake may have caused an accumulation of pollutants which have shown

up in the algae. The product is no longer sold in the United States. This has resulted in the development of several aquaculture companies in the U.S. to fill the demand.

Spirulina has shown up regularly in health food fad periodicals and has been reported to cure everything from hangovers to A.I.D.S., none of which is based on scientific evidence, but then this is not unusual for health food faddists. There is however extensive scientific evidence supporting the exceptional quality of this algae as a protein source and it's high content of B-vitamins and essential fatty acids. The protein quantity and quality is truly remarkable at 50% to 70% of the total mass, and up to 85% digestible. Comparing this algae to other vegetable or single cell protein (SCP) sources is unfair due to it's superior quality, and can only be compared to things such as powdered milk or dried eggs.

The nutritional value (Chung) and detailed composition of *S.platensis* (Durand-Chastel) are well documented. The later publication surveys the composition of *S.maxima* and *S.platensis* and reports maximum and minimum values for ash, salts, metals, protein and amino acids, fatty acids and nonsaponifiable lipids, sterols and carotenoids, carbohydrates, vitamins and heavy metals. Many of these values are included in table 1 in the appendix. A more in depth study on the digestibility and nutritional studies on

rats in comparison with casein is given by Clement. The nutritional analysis of *S.maxima*, raw and in various preparations, focus on protein, aminoacid composition and vitamins (A, B1, B2, B6, B12,and C). Comparative rat studies fed with Spirulina as a sole protein source gave a net protein utilization (NPU) of 45-50%, digestibility (D) of 76%, biological value (BV) of 60-65% and a chemical score of 43-56%. (NPU=BVxD) This is relative to eggs with a chemical score of 100 and milk with a score of 78. Weight gain studies showed that rats grew equally well on *Spirulina* as on casein. The authors also find that the essential amino acids lysine and methionine are not limiting as is found with most vegitable sources.

Although it grows in filaments, Spirulina is classified as a SCP product. It is far superior to other SCP's for the aforementioned reasons and that it has a relatively low nucleic acid content which is a major drawback of protein supplements such as yeast or *Lactobacillus*. The B-vitamin and fatty acid composition are an extra bonus. Extensive studies have been done on the effects of a *Spirulina* diet either as a supplement or sole source of nutrition. The possibility of toxic or mutagenic components has also been pursued. In no case have any adverse effects been reported.

There are several biomolecules produced in moderate to high abundance by this algae that are of current or future

interest. Cohen has evaluated the fatty acid content of 19 strains of spirulina and their response to environmental stimuli. All strains studied contained gamma-linolenic acid (GLA) in the range of 0.7 to 1.4% of the total biomass. GLA has been reported to have beneficial effects such as: the lowering of blood cholesterol, treating atopic eczema, and positive effects on premenstrual syndrome, heart disease, Parkinson's disease and multiple sclerosis. In addition it has positive effects on fish reproduction when used as a feed in aquaculture (Oceanic Institute, personal communication).

Some carbohydrates have shown some utility in cancer therapy. A patent on an antitumor carbohydrate from *S.subsalsa* designated y and k spirulinan has been applied for (Japan, Yamaguchi). Indeed two unusual carbohydrates have been isolated from *S.platensis*, however a connection has not been made between these two studies. The biliproteins are of use as a nutritional food coloring (although there doesn't seem to be a big calling for cyan colored food) and recently as a fluorescent tag in immuno-assay conjugates. Also there is a publication on the regression of hamster cancer by *S.platensis* extracts containing pigment (Schwartz).

Other clinical uses of Spirulina involve the treatment of the obese by spirulina diets (Becker) and the treatment of gastric and duodenal ulcers (Fica).

The dried organism itself is showing up in more and more prepared foods, primarily health foods. It can be found in candy bars, natural herbal stimulants, vegetarian meat substitutes and as a dried powder. It has yet to break into the markets dominated by soy, wheat and yeast, and will remain excluded until; 1) a better controlled and more cost effective way of producing the algae can be designed, and 2) a more appealing way of preparing and marketing the product is developed such that it may compete with other protein supplements. These two factors go hand in hand, and it is up to the future commercial suppliers to come up with the answers. These answers will only come when corporations take on development of the product with the forethought and seriousness of any other food product rather than simply production of biomass.

INTRODUCTION

CYANOBACTERIA TAXONOMY

Cyanobacteria is now the more often used term for the photosynthetic prokaryotes previously referred to as "blue-green" algae. They are virtually ubiquitous in nature and occupy an essential niche in the turnover of inorganic nitrogen and carbon. They can be found in virtually any environment that contains water, air and mineral. This description may be more limiting than vague in that the phylum/division is adaptive to the most extreme of environments. Generally, where there is water and sunlight, one will undoubtedly find cyanobacteria. The organism(s) are characteristically of a blue-green or "cyan" color which arises from the production of a photosynthetic pigment known as phycobilin associated with a biliprotein, collectively known as a phycobiliprotein.

The morphology of this group of bacteria is varied; single cells in the sub micron level to filaments several millimeters long and tens of microns in diameter, colonies as either disorganized masses or semi crystalline, filaments either straight or branched, outer sheaths that are almost nonexistent or occupy a major fraction of the cell mass, and differentiating or non differentiating. This last category usually refers to the process of heterocyst formation for nitrogen fixation (i.e. whether

the species can reduce diatomic nitrogen to organic nitrogen). There are even more fundamental characteristics for segregation such as: heterotrophic or autotrophic (faculative or obligate), photoadaptation (at least three subdivisions), osmotolerance (marine, brackish, or fresh water), motile or non motile, etc.

One may ask how this all relates to taxonomy. Currently there are three popular competing taxonomic terminologies combined with over a half a dozen collection agencies with their own numbering systems for each isolate (remember they are a most adaptive organism) making the study most confusing. Amazingly enough there remains a good deal of consistency in the identification. A single species may be labeled with three or more names and still be recognized by separate dogmas.

There has been adopted in recent years, a system of classification that segregate the cyanobacteria into five categories. Table #1 summarizes this classification system. The organisms most often used in genetic and molecular biology studies are found in sections I, III and IV. There are many practical reasons for the predominant use of these species however the earlier studies on nitrogen fixation in cyanobacteria has probably more to do with the popularity of one species over another than anything else.

TABLE 1
Cyanobacterial Taxonomic Divisions

Sec	Basic morphology	Reproduction	plane of division	Order(Family)	Representative genera
I	unicellular	binary fission	single	Chroococcale	Gloeobacter,Synechococcus, Anacystis,Agmenellum
II	unicellular	budding multiple fission	two or more	Chamaesiphonales	Chamaesiphon, Dermocarpa
II	unicellular	budding multiple fission	two or more	Pleurocapsales	Xenococcus, Pleurocapsa
III	filamentous nondifferentiating	tricome fragment hormogonia	single	Nostocales (Oscillatoriaceae)	Pseudanabaena, Spirulina,LPP group
IV	filamentous heterocystous	tricome fragment akinetes	single	Nostocales (Nostocaceae)	Anabaena,Nostoc,
				(Rivulariaceae)	Calothrix (Fremyella)
V	branched filaments heterocysts	tricome fragment hormogonia and akinetes	two or more	Stigonematales	Mastigocladus,Ficherella

ref; "The Blue-Greens" by P. Fay

As mentioned earlier there are several additional methods of segregating the cyanobacteria aside from gross morphology. These range from the more obvious such as the species capacity to fix nitrogen, to the sophisticated analysis of rRNA fragment comparison. Somewhere in the middle there is photoadaptation. This characteristic has been referred to in a variety of applications, mostly biochemical, however in the case of using it as a taxonomic indicator, it most often refers to biliprotein (pigment) turnover under varying light conditions or chromatic adaptation. The details of those processes will be discussed later. The classifications are:

I) no biliprotein response to light quality

II) PE synthesis is induced by green light, PC is constant

III) PE is induced by green light, repressed by red, PC is repressed by green and induced by red.

Biliproteins are by no means the only cell components that show light response but they are the only components that allow for differentiation among species.

To reiterate some initial statements about taxonomy, the accepted classifications are based on morphology from wild isolates with little or no biochemical or genetic support. Where a connection has been attempted the results at best confuse and often contradict the system. Without some concerted effort to resolve these problems,

genetically or otherwise, we are stuck with a shaky system of identification that might well be impossible to improve upon.

CYANOBACTERIAL GENETICS

The work on cyanobacterial genetics and molecular biology started late relative to other prokaryotes. Interest in these photosynthetic bacteria arose in the late 60's with the development of the tools of molecular biology and a great commercial and political interest in nitrogen fixation in agricultural systems, both domestic and international. It had long been known that most cyanobacteria are nitrogen fixers and that their photosynthetic metabolism was very similar to higher plants. The cyanobacteria thus became an excellent vehicle for the study of these basic processes of phototrophic metabolism. In addition there are several adaptive processes that became of interest: photoregulation, shift from aerobic to anaerobic metabolism and differentiation from vegetative cells to heterocysts, and of course, the isolation and construction of various recombinant vectors.

However only a few species have made their way into the literature probably due in part to their historical use as model organisms, but more so due to their ability to be manipulated by traditional microbiological, genetic and

molecular techniques, with minimal modifications. These species are *Anacystis nidulans* R2 or *Synechococcus* ATCC6301, *Agmenellum quadruplicatum* or *Synechococcus* ATCC7002, *Nostoc* PCC7524 and 8009, several *Anabaena* and *Synechocystis* strains and more recently *Fremyella* (also called *Calothrix*). The later two are less studied due to the filamentous nature of the organism which complicates selection of recombinants or mutants. All of these strains have been selected for qualities necessary for use in recombinant research. They possess endogenous plasmids that could be reconstructed into hybrid shuttle vectors for use in *E.coli*, have some mode of transformation (Porter), conjugation (Flores), or transfection (Sammi) into the host, stable expression of the vector, a mode of mutagenisis either chemical, UV or by transposon, and the ability to grow on selective media in a reasonable amount of time (marker selection may take from 10 to 30 days).

In regard to transformation, which is by far the most common mode of gene transfer, most, if not all of the strains, are naturally competent and can be transformed by adding the DNA directly to the growth media, usually BG11. Several studies have been done evaluating various parameters such as growth phase, light or dark conditions, cell density, DNA concentration and calcium shock (Devilly, Chauvat), however little of this has seen any practical use in the literature. As mentioned earlier, the only other

method in common use aside from physiologic transformation, is conjugation of *E.coli* bearing hybrid vectors with filamentous cyanobacteria using a third helper strain of *E.coli* (Wolk).

The type of vectors used are essentially no different than any other system in that, a cyanobacterial DNA;*E.coli* replicating hybrid plasmid is constructed and selection is through antibiotic resistance (usually chloramphenicol). The variety of recombinants is growing every day and a broad range of hybrid plasmids, expression vectors, cosmids, and transposable markers in vectors have been constructed (Stevens). In addition there is some evidence that various types of transformants can be constructed out of homologous recombination using a marker DNA flanked by genomic DNA. The stability of these transformants is dependent upon size and type of flanking DNA (Kolowsky,Lightfoot,Williams).

One aspect that has received surprisingly little attention is that of DNA modification in conjunction with species specific restriction systems. This is undoubtedly the main barrier to transformation of *Spirulina*. As mentioned, it appears that the work on cyanobacteria has been restricted to those strains that these problems have been fortuitously sidestepped. There are two relevant studies that have investigated these aspects. Whitehead and Brown developed a simple method for screening bacteria for

type II restriction enzymes that is applicable to cyanobacteria. Rabindranath later reported on the quantitative estimation of the degree of methylated bases in cyanobacterial DNA by restriction analysis. Application of these methods to the as yet unexploited cyanobacteria may lead to more versatile and broad range vectors.

Currently work on the mapping and characterization of the recA gene in several cyanobacteria is progressing with the hope that it can be stably repressed in desirable strains (Herrero,Murphy) allowing for more sophisticated recombination experiments, such as site directed mutagenesis and complementation or expression of foreign genes.

Regarding the later, there is a fair amount of progress already on the use of signal genes such as beta-galactosidase and luciferase in combination with various cloned promoter sequences demonstrating promoter strength and regulation in cyanobacerial vectors (Elhai in press).

THE PHYCOBILIPROTEINS

The light collecting apparatus of the cyanobacteria are referred to collectively as the phycobiliproteins. These protein chromophore structures have been well studied in regards to their protein and chromophore makeup, assembly and more recently their genetics and expression

(McColl). There are three major categories of biliproteins: the allophycocyanins, the phycoerythrins and the phycocyanins. Each has it's own characteristic absorption spectra. The combination of these chromophores allow the structure to collect light from a range of wavelengths. The biliprotein C-phycocyanin is probably best understood due to it's ubiquitous nature and high abundance. It is this protein pigment that gives the cyanobacteria their characteristic cyan or blue-green color. In the case of *Spirulina* only the C-Phycocyanin and allophycocyanin are present.

The general makeup of the antennae structure is a core structure of allophycocyanin that is attached to the thylakoid membrane adjacent to the chlorophyll-a site. Stacked upon this core is a collection of six rods composed of hexameric biliprotein disks. Each disk is composed of three dimeric subunits ($\alpha 3\beta 3$) and these discs stack upon one another to form the rod (Fig1). Many cyanobacteria have rods that are a combination of phycocyanins and phycoerythrins. The phycoerythrins being most proximal and the phycocyanins lying between those and the allophycocyanin core. The relative abundance of each chromophore is dependent on the available light, thus allowing the organism to optimize it's absorption spectra. The entire antennae structure is assembled with the aid of four to seven linker peptides each with it's own function,

that is, the assembly of discs, rods, cores, etc. The following table gives some examples of the size range of these linker peptides.

TABLE 2

MOLECULAR WEIGHT OF PRINCIPLE LINKERS BY SDS PAGE (δX1000)

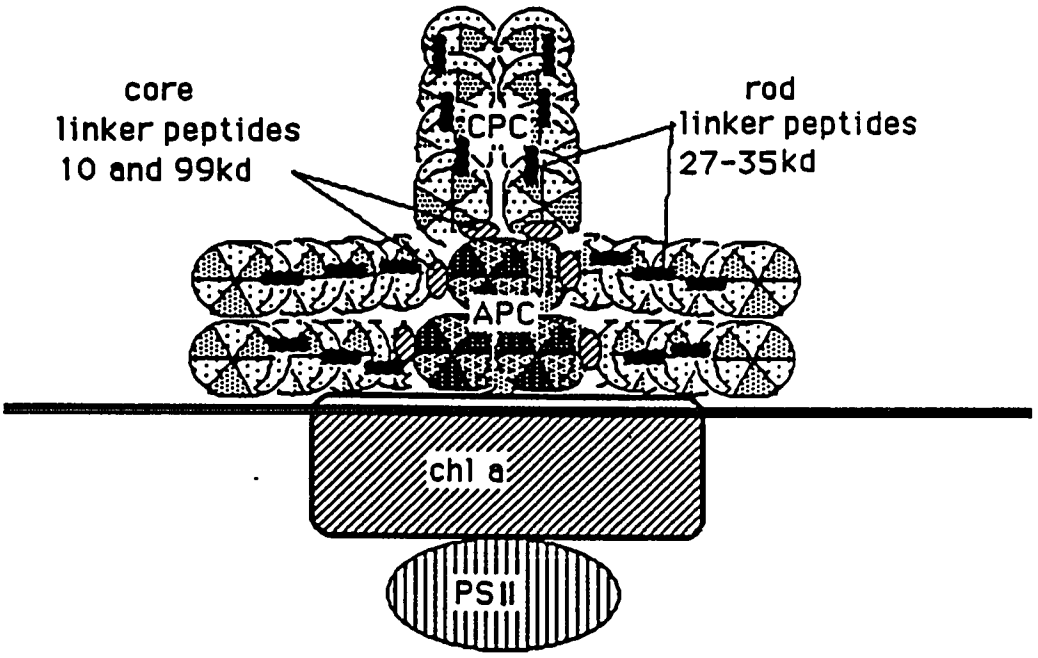
Anabaena var.	Nostoc	Synech.6301	A.nidulans	Pseudana.
120	95	99	75	94
32.5	34.5	33.5	33	34.2
30.5	34	31.5	30	32.6
29	32	30.5	-	31.3
27	29	27	27	27.4

ref: "The Phycobiliproteins" McColl ed.

Figure 1.

The Phycobiliprotein Model

An artistic rendition of the antennae structure composed of hexameric discs,rods and the core assembly lying adjacent to the chlorophyl a site of the thylakoid membrane.



In general the high and low molecular weight linkers are involved with core attachment and assembly, and the 30-35kd linkers in rod assembly. As with the biliproteins, linker composition varies with growth conditions particularly with light quality.

The biliproteins have also been found to possess carbohydrates (glucose and mannose) attached to several subunits particularly the linker peptides and the core anchor protein. Carbohydrates can amount to as much as 2% of the total molecular mass of the bilisome. Their function is uncertain, but they may be involved in membrane assembly and glycogen turnover in response to stress (Reithman).

All of the pigments are photoregulated to some degree. Photoregulation of the biliproteins varies with the organism and is also spectral dependant, but in general, expression is inversely related to light intensity. More specifically, the phycoerythrins and phycocyanins respond to spectral wavelengths that correlate with their given chromophore. This is referred to as complementary chromatic adaption. That is, the synthesis of each biliprotein is regulated primarily by light within it's absorption maxima. Early work on protein profiles of biliprotein extracts from numerous strains demonstrated distinct patterns of response. Cyanobacteria can be lumped to three general categories according to the way they respond to light: 1)nonadapting, 2) only PE adapts and 3) both PE and PC

adapt (Bryant and Cohen-Bazire). In the case of some of the group 3 species, two sets of C-type PC subunits could be observed indicating two genes. Later work found that this regulation is operative on the transcriptional level and has a response time on the order of minutes. The amount of biliprotein may fluctuate as much as 50% without noticeable change in growth rates. With biliproteins comprising 15% or more of the total soluble protein, this is a significant protein turnover for the cell during photoadaption (Boussiba).

The actual mode of control that links light energy to transcriptional control has not yet been determined in the cyanobacteria, however there is speculation that a phytochrome like system may exist analogous to that of higher plants (Hersey,Quail). If indeed such a system does exist in the cyanobacteria it would have to be multiple regulators or have changing DNA binding affinities under varying light conditions in order for it to differentiate regulation of multiple genes.

What has been determined is that part of the regulation has to do with multiple genes that are either constitutive or inducible with a polycistronic message that encodes some of the rod linker peptides. Under red light adaption, the read through is terminated prior to the linker genes, effectively reducing rod length. The genomic organization of the biliproteins has been deduced in

Fremyella (Grossman, Conley). The APC gene cluster is arranged in the order of: linker peptide-ORF-alpha subunit-beta subunit-linker peptide, and is upstream from the C-PC cluster containing the inducible gene; beta subunit-alpha subunit-linker peptide followed by the constitutive gene; beta subunit-alpha subunit-30kD ORF. The PE gene has not yet been linked. Northern hybridization to preps from adapted cells shows processing of the messages from the APC gene. A large premessage is processed to a smaller 3kb and 1kb message and the 3kb to a 1kb as adaption progresses without apparent changes in hybridization signal intensity. The C-PC message shows a simple quantitative response being high in red light and low in green light. Analysis of the transcript sequence has shown a potential for elaborate hairpin structures in the prestructural region of the linkers. This region may be involved in the regulation of the bilisome. Similar results on *Anabaena* (Belkamp and Haselkorn) lead to the proposal that the regulation of the linker peptide ratios control bilisome rod length and therefor light capture efficiency.

Tandeau de Marsac, et.al., have found similar results in *Calothrix* and have proposed that the co-transcription of an element downstream form the C-PC cluster may be involved with the differential rates of expression. They have found that mutation of this region, called orfX, represses C-PC expression and PE synthesis is stopped. It is proposed that

this product may be acting on the secondary structures of the C-PC cluster transcripts. An alternative explanation is that orfX codes for a protein modifying enzyme that prevents degradation of the biliprotein. This has been postulated by others (Boussiba) as the control mechanism for PC levels.

The first cloning of the C-PC gene appeared as simultaneous publications (DeLorimier, Pilot), both from *Agmenellum* using oligonucleotides based on different regions of the protein sequence. Subsequently the C-PC gene from numerous other species have been cloned using either heterogolous probes or synthetic oligonucleotides. Considerable homology has been found in all cases and often enough homology exists between the different biliproteins that cross hybridization does occur (Conley, Zilinskas). The general consensus is that all biliproteins arose from a common ancestor and have been highly conserved throughout evolution with few exceptions.

Oddly enough there is no sequence data for *Spirulina* biliproteins. In fact the only sequence data obtained for *Spriulina* is a partial amino acid sequence of cytochrome-c (Gomez-Lojero). The APC and C-PC proteins have been isolated and partially characterized. The C-PC dimer in *S.platensis* has an absorption maxima at 620nm (E=73) and a molecular weight of 18kD for the alpha subunit and 23.5kD for the beta. The APC dimer absorbs at 655nm (E=58) and the

alpha and beta subunits weigh 18kD and 20kD respectively (Boussiba and Richmond). Amino acid analysis of the dimeric protein is given in table 3 with some comparative data. A short compiled list of N-terminal amino acid sequences is given in table 4 with some original data on *Spirulina platensis*.

The only other data that is an article describing the preparative isoelectric focusing of biliproteins. The pI for C-PC subunits from *S.geitleri (maxima)* are 5.0 and 5.6, alpha-beta, relative to *M.laminosus* at 6.1 and 5.0 (Kost-Reyes).

TABLE 3

TOTAL AMINO ACID ANALYSIS OF SOME C-PC DIMERS

a.a.	S.platensis	A.nidulans	Ag.quad.
lys	13	13	13
his	1	1	2
arg	18	18	19
asp	31 (21)*	23	20
thr	18	19	26
ser	24	19	26
glu	30 (14)*	14	16
pro	8	9	9
gly	27	26	25
ala	56	58	50
cys	4	4	5
val	18	20	23
met	9	4	9
ile	19	16	14
leu	29	36	28
tyr	16	15	16
phe	12	12	11
trp	(1)*	1	1
gln	(10)*	7	12
asn	(16)*	17	16
total	333 (334)*	338.	334

* inferred from data

TABLE 4

COMPILED N-TERMINAL SEQUENCE DATA FOR C-PC SUBUNITS

BETA

Spirulina platensis*	mfdaftkvvsqadt gefl
Synechococcus 6301	tfdaftkvvaqadargefl
Anabaena variabilis	gldvf kv qad rgefl
Mastigocladus laminosus	aydvftkvvsqadsrgefl
Cyanidium caldarium	mlbafakvvaaabargefk
Agmenellum quad.	mfdiftrvvsqadargefi
Fremyella diplosiphon	mldaftkvvsqadtggayi

ALPHA

Spriulina platensis*	mktplteavsiadsqg flst
Synechococcus 6301	sktpl eavaaab g
Anabaena variabilis	vktpiteaia ad qgrfl
Mastigocladus laminosus	vktpitdaiaaadtqgrfl
Cyanidium caldarium	mktpiteaiaaabarg
Agmenellum quad.	mktplteavaladsqgrfl
Fremyella diplosiphon	mktplteavatadsqgrfl

*data from this work

MATERIALS

1. Frequently used buffers

TAE (tris-acetate-EDTA) for agarose gels

Working solution

0.04 M Tris-acetate

0.001 M EDTA

pH 7.8

50X stock

per liter;

Tris base	242 g
glacial acetic acid	57.1 ml
0.5 M EDTA	100 ml

20X SSC (sodium chloride-sodium citrate)

per liter

NaCl	175.3 g
Na citrate	88.2 g

adjust pH to 7.0 with concentrated NaOH

Restriction enzyme buffers (made as 10X stock)

	final concentration (mmol/l)				
	A	B	L	M	H
Tris acetate	33				
Tris-HCl		10	10	10	50
Mg-acetate	10				
MgCl2		5	10	10	10
K-acetate	66				
NaCl		100		50	100
DTE			1	1	1
DTT	0.5				
EtSH		1			
pH	7.9	8.0	7.5	7.5	7.5

Restriction enzymes used grouped by buffer

A	B	L	M	H
Acc1	Ava1	HpaII	BglII	ClaI
AvaII	BamHI	KpnI	Tth111I	EcoRI
HpaI	HindIII	BstYI(XhoII)		PstI
SacI			XmaI	PvuI
Sau3AI				SalI
SmaI				XbaI
				XhoI

2. Frequently used enzymes

Restriction Enzymes, ligase, etc. were purchased from

Boehringer Mannheim Biochemicals (BMB)
P.O. Box 50816
Indianapolis, IN 46250

Bethesda Research Laboratories (BRL)
P.O. Box 6009
Gaithersburg, MD 20877

New England Biolabs (NEB)
32 Tozer Rd.
Beverly, MA 01915-9990

3. Frequently used Materials

Mini-spin columns

Worthington Biochemical Corp.
Halls Mill Rd.
Freehold, NJ. 07728

Centricon-10 Microconcentrators

Amicon Division, W.R.Grace & Co.
17 Cherry Hill Dr.
Danvers, MA 01923

Nitrocellulose filters

Schleicher & Schuell Inc

Keene, NH 03431

Zeta-Probe filter

BioRad Laboratories

1414 Harbor Way South

Richmond, CA 94804

NENsorb-20 columns and radioisotopes

New England Nuclear

549 Albany St.

Boston, MA 02118

Sea-Plaque agarose

FMC BioProducts

5 Maple St.

Rockland, ME 04841-2994

CsCl and "Sequenase" kit

United States Biochemicals

P.O. Box 22400

Cleveland, OH 44122

also BMB

4. Plasmids received from outside laboratories

From:

Dr. Tami Pilot

Dept of Zoology

University of Texas

Austin, TX. 78712

pTP1; a 4.2kb clone of the CPC gene from *Agmenellum quad.* in pUC8

From:

Dr.C.A.M.J.J. vandenHondel

Medical Biological Laboratory

TNO, P.O. Box 45

2288 GJ Rijswijk

The Netherlands

pRI46 : a Tn901 transposon bearing plasmid

pPUC29: a *E.coli:A.nidulans* cosmid vector

pTH225: a *A.nidulans* methionine:pPUC29 recombinant

pACYC184: a *E.coli* plasmid vector

From:

Dr. B.Bohlool

Dept of Microbiology

University of Hawaii at Manoa

Honolulu, Hawaii 96822

pRMR2 : a nif clone from *Klebsiella*

From:

Dr. J. Hunt

Dept. of Genetics

University of Hawaii at Manoa

Honolulu, Hawaii 96822

pIBI25 : sequencing vector

pZF18U : " "

From:

Dr. O. Tiboni

Dept. of Genetics and Microbiology

University of Pavia

27100 Pavia,Italy

pSP301 : a 4.7kb clone of RUBPCase from *Spirulina platensis* in pBR322

From purchased kits or available in lab:

pUC8 : carries AMP marker and lacZ/polylinker region

pUC12 : "

pUC-4K : " with a kanamycine insert

M13mp18

TABLE 5

STRAINS OBTAINED FROM THE OCEANIC INSTITUTE 5/31/83

SP3-416	Spirulina platensis	(origin ?)
SM-20	S. maxima	(Mexico)
AP-14	Arthrospira platensis	(Thomas 4-16)
SP-19S	S. platensis marine	(Jamacia)
SP-19	S. platensis brackish	(Jamacia)
AP-4	A. platensis	(origin ?)

STRAINS OBTAINED FROM UNIVERSITY OF TEXAS EL PASO 12/20/84

UTEX 770	S. sp.	soil
UTEX 2179	S. sp.	soil
UTEX 552	S. major	ES media
UTEX 1954	S. subsalsa	ES
UTEX 1318	S. subsalsa flos aqua versicolor	1/4soil+ES
UTEX 2342	S. maxima	O.T.
UTEX 2340	S. platensis	O.T.
UTEX 1926	S. platensis	ES
UTEX 1928	S. platensis	ES

TABLE 5 (cont.)

UTEX 1932 Nostoc linckia BG

UTEX 1552 Anabaena spirodes BG

UTEX 581 Plectonema boryanum BG

UTEX 594 Plectonema boryanum BG

UTEX 426 Phormidium luridum BG

UTEX B377 Anabaena variabilis BG

UTEX 485 Plectonema boryanum BG

UTEX 1037 Nostoc muscorum BG

UTEX 2268 Agmenellum quadruplicatum ES

UTEX 2434 Synechococcus leopoliensis (Anacystis nidulans) BG

STRAINS OBTAINED FROM THE UNIVERSITY OF HAWAII

ATCC 27180 Synechococcus

ATCC 18200 Plectonema

ATCC 27892 Anabaena

ATCC 29344 Pseudanabaena

ATCC 27344 Anacystis

ATCC 27347 Nostoc

HB101 E.coli

MM294 E.coli competent cells CloneTech

NM522 E.coli

JM103 E.coli

METHODS

Note: Under all categories several methods are given. No one method worked for all strains, each application being determined empirically. In general, all procedures are listed in order of frequency of use.

ALGAL CULTURE TECHNIQUES

ALGAL GROWTH MEDIA

MACRO (g/l)

	O.T.	BG-11	ASN-III
$NaHCO_3$	16.8		
Na_2CO_3		0.02	0.02
K_2HPO_4	0.5	0.04	0.029
$NaNO_3$	2.5	1.5	0.75
K_2SO_4	1.0		
KCl			0.5
NaCl	1.0		25.0
$MgSO_4.7H_2O$	0.2	0.075	3.5
$MgCl_2.6H_2O$			2.0
$CaCl_2.2H_2O$	0.04	0.036	0.5
$FeSO_47H_2O$	0.01		
EDTA	0.08	0.001	0.0005
Citric acid		0.006	0.003
Fe/NH_4 cit		0.006	0.003
pH	@8.5	7.1	

MICRO (X1000)

A5 (g/l)		B6 (mg/l)	
H_2BO_3	2.85	NH_4VO_3	23
$MnCl_2.4H_2O$	1.81	$K_2Cr_2(SO_4)_4.24H_2O$	96
$ZnSO_4.7H_2O$	0.22	$NiSO_4.7H_2O$	47.85
$CuSO_4.5H_2O$	0.08	$Na_2WO_4.2H_2O$	17.94
MoO_3	0.015	$Ti_2(SO_4)_3$	40.0
		$Co(NO_3)_2.6H_2O$	44.0

Micronutrient substitutions;

NH_4VO_3 (23mg) -> $NaVO_3$ (24mg)

$CrK(SO_4)_3.12H_2O$ (96mg) -> $CrNH_4(SO_4)_2.12H_2O$ (100mg)

$Ti_2(SO_4)_3$ (40mg) -> $Ti(NO_3)_3$ (48.8mg)

$Co(NO_3)_2.6H_2O$ (44mg) -> $CoAc_2.4H_2O$ (37.5mg)

Liquid media

Stock solutions of the above minerals are diluted to the appropriate concentration in DD H2O and filter sterilized.

Solid media

A 2X concentrated sterile media is prepared and mixed with an equal volume of 2% agar in DD H2O (autoclaved) and immediately poured into petri dishes.

Axenic Culture for Cyanobacteria

I

The culture to be purified is grown to early log phase (OD560=0.2-0.5) and harvested by centrifugation. The pellet is resuspended in sterile media, sonicated briefly and repelleted (3X). The pellet is washed until the supernatent is clear of pigment. The cell suspension is then treated with U.V. (7min,20W,38cm)and/or antibiotic and the serially diluted in test tubes containing appropriate media. The innoclum is then allowed to grow for several generations. The culture appearing at the highest dilution is selected for further work. This process is repeated as necessary. Purity is determined by visual inspection and plating on nonselective rich media (eg. LB).

II ref.Ogawa and Terui

Cells are grown in OT media with agitation by aeration at 0.25 vol/min,Temperature 35 C, and illumination with a tungsten lamp (100 W) at 2.5-3 klux. Growth is monitored by optical density at 560nm.

1) grow cell suspension at high dilution to limit contamination. Harvest by filtration on habutae silk (or GFA glass fiber filters)
2) wash cells with basal media, resuspend and filter
3) wash,resuspend, and homogenize with sonicator. Length of sonication must be determined empirically depending on

the apparatus used. (Using a Labline "Ultratip"sonic at a power setting of 80 filament fragments 4-20 cells could be obtained in three cycles.) Filter. Repeat 3X.

4) spread cells in a thin layer in a petri dish and expose to U.V.,20W,38cm, for 7 min. with agitation.
5) stand in the dark for a few hour to prevent photoreactivation.
6) dilute to one cell per drop(~0.04ml)
7) inoculate testubes containing 10ml basal media with one drop of treated cells.

Notes on method II

(from author)

a) steps 3 and 7 are critical
b) approximately one in fifty inoculum are viable
c) doubled growth yields obtained in mixotrophic culture: basal+0.1%glucose+0.1%peptone
d) mixotrophic lysis occurs at subcritical inocula levels when transferring from autotrophic to mixotrophic (0.5%glucose) media.
e) effective mixotrophic carbon sources are glucose,peptone,arginine,aspartic acid,leucine,proline, TCA organic acids;acetic,butyric,tartaric and maleic acids.

(Personal Notes)

f) sonication in a waterbath sonicator in the presence of

Triton-X-100 (1 drop/100ml) aides in the removal of bacteria stuck to the cell wall.

2) five washings appears to be the practical limit in these procedures

III ref. Stein

1) dissolve penicillin G, (100mg) and streptomycin SO4, (50mg) in 10ml of H2O
2) add chloramphenicol,10mg in 1ml EtOH
3) filter sterilize
4) prepare a series (6) of 125ml flasks containing log phase cells diluted 1ml in 50ml of media
5) add 3,2,1,0.5,0.25,0.125ml of antibiotic solution to each flask.
6) incubate with aeration 24-48hrs then check for bacteria by plating. Repeat as necessary.

IV

An actively growing culture is harvested either by filtration or centrifugation, resuspended in sterile basal media, briefly sonicated and reharvested. This cycle is repeated 2X. The algal suspension is then serially diluted in complete media and placed under light/dark growth conditions for 3-4 weeks. The growing culture at highest dilution is selected for further use or recycled.

Note: In the case of all Spirulina strains investigated strains were maintained in the presence of kanamycine at 50 ug/ml which helps to prevent contamination of cultures.

Tolerence Tests

To evaluate resistance and tolerance to antibiotics and metals, gradient plates were made as follows. Nutrient agar was prepared as previously described. To one batch was added the test material to maximum test concentrations. Approximately 10 ml of this solution is poured into a petri dish set on a slant such that a minimum of the bottom is exposed, and allowed to harden. The plates are then leveled and an over layer of nutrient agar is poured to level. Plates are stored for 48 hrs before using.

Algal liquid cultures are spread on the surface of the test plates and tolerance scored, after an appropriate time, by the line of active growth on the plates. A linear gradient from one side of the plate to the other is assumed.

Growth Rates

Growth rate and cell density are most readily determined by optical density at 600 nm. At this wavelength both turbidity and absorbance by chromophores are factors. A linear relation between OD and log [cells/ml] is observed

in the range of 0.1 to 0.7 OD600. At OD=0.5 the cell density is approximately 10^8 cells/l. This was determined by measuring OD of a serially diluted culture, counting the number of filaments in an aliquote, counting the average number of cells per filament and calculating cells/l. In a separate analysis, linearity was observed in the range of 0.1 to 1.9 OD600 with a slope of approximately 450 when plotting mg/l dry weight vs. OD 600, for outdoor raceway cultures.

Doubling rates under 16 hr-on/8 hr-off fluorescent light, 32 degrees C, in O.T. media was found to be near to the documented value (Ciferri, 86) of 0.2-0.3 per day.

Harvesting

Several methods of harvesting are;

1) Vacuum filtration onto glass fiber filters. Although the most common method of harvesting, recovery is not 100% and care must be taken not to scrape the filter when removing the cells in order to prevent the inclusion of glass fibers in the cell mass which may interfere with further isolations.

2) Centrifugation at 2500 g. for 10 min. after brief sonication. The sonication is necessary to disrupt gas vacuoles that prevent pelleting of cells. The duration of sonication is dependent on the apparatus used. A bath sonicator will require as long as 1 min., while a probe

sonicator at 50% power will require 3-10 sec. While some cell disruption does occur, the loss is negligible.
3) Scraping the cells directly from plates. This is a simple way of recovering a small amount of cells from available plates where only a small preparation is required.

Mutants

ref. O.Riccardi (1981)

"Amino acid analog resistant mutants"

1) cultures grown to early stationary phase (2,5x10^4 filaments per ml)
2) treat with nitrosoguanine at 100ug/ml for 30 to 60 mins or to 0.1-1.0% survival.
3) wash free of mutagen by filtration
4) plate on basal media in 1.5% agar with 1g/l peptone
5) incubate for 10 days under continuous illumination
6) replica plate with a velvet pad to minimal media with analog supplement at various concentrations.

Mutant frequency reported as 1x10^-7 for 5-fluro-tryptophan and 1.2-7.1x10^-6 for other (3) analogs for *S.platensis*

Mixotrophic culture

1) From a stock culture growing at log phase aliquotes were

taken and placed in 10 ml glass test tubes with fresh media containing one of 16 amino acid at 20 ug/ml.

2) growth was checked approximately every 10 days by optical density at 600 nm.
3) the same procedure was applied to carbohydrates
4) substrates tested were; ala,arg,asp,glu,his,ile,leu,lys, met,phe,pro,ser,thr,trp,tyr, val, glucose, mannose, galactose, mannitol, fructose, ribose, xylose, D and L-arabinose, lactose, sucrose and trehalose

Restriction Enzyme Analysis

I

This method was an adaptation of a method used in Dr. Richard Manshardt's lab, Dept of Agriculture,Univ. of Hawaii where we were using the extraction procedure for isozyme analysis in plants. The remainder was developed by the author.

1) approximately 27g of frozen wet packed cells (SM-20) were homogenized in 40 ml of extraction buffer

Tris pH=7.3	130mg
sucrose	3.42g
EDTA	30mg
ascorbic acid	84mg
sodium bisulfite	52mg
DIECA	105mg

mercaptoethanol 100ul

H2O to 50ml

homogenization by sonication at full power for 1min on ice.

2) centrifuge in Sorvall 34, 10K rpm, 0 C, 30min

3) supernatent poured off and made 70% saturated with ammonium sulfate on ice

4) centrifuge 10K rpm, 0 C, 30min

5) pellet resuspended in 20ml elution buffer

KH2PO4 20mM

EDTA 0.1mM

MgCl2 0.2mM

6) load on CM cellulose column (25x2cm) and eluted with a NaCl gradient 0-0.6% in elution buffer at 4 C, 0.15ml/min

7) fractions collected every 2.5ml 50 fractions total

8) every 3rd fraction lyophylized, resuspended in 1ml elution buffer and dialized against same.

9) an 18ul aliquote was taken from each fraction to which was add 2ul of 10X 'M' buffer and 2.5ug of plasmid DNA (pBR322,pACYC184, or pPUC29) and incubated at 37 C for 0.3-3.0 hrs.

10) each reaction was the run on a 0.7% gel and visualized with ethidium bromide.

II

ref.Whitehead and Brown (1985)

Preparation of cell free extract

"...A fresh bacterial pellet from 4ml culture was resuspended in 200ul 25% (w/v) sucrose 50mM Tris-HCl, pH7.5. Lysozyme (a fresh 1 mg/ml solution) and EDTA (500mM,pH8.0) were added to final concentrations of 0.1 mg/ml and 10mM respectively. The mixture was incubated at 0 C for 2min. MgCl2 and Birg-58 (Triton-X 100) were added in that order to the required final concentrations (50mM and 0.5% respectively) and the sample was incubated at room temperature for 15min. The sample was centrifuged at 10,000xg for 10min at 40 C and the supernatant was assayed for endonuclease activity."

Samples (1.5ul) of the supernatant were incubated with either lambda or pBR322 DNA (0.25ug) in 25ul of buffer. (The buffer used was generally 'M' buffer however other buffer systems were used on certain species eg. 'H' buffer was optimal for *Spirulina* species). Incubations were for 0.5-3hrs at 37 C. Incubations with no added DNA insured that any endogenous bacterial plasmids did not confuse interpretation of results. Reaction mixtures were analyzed by horizontal agarose gel electrophoresis.

A scale-up of this procedure was also done and the product supernatant was fractionated on molecular sieve (Sephadex G-100) and ion exchange resin (DEAE-Sephadex) in

an attempt to separate possible restriction enzymes (Kawamura). A phosphate-glycerol buffer was used in both cases with a salt gradient from 0 to 0.3M added in the case of the ion exchange resin.

TRANSFORMATION OF *Spirulina sp.*

I U.V. Induced transformation

ref.Dzelzkalns and Bogorad

1) strains UTEX 2340 and 2342 were grown to early log phase (OD600=0.5-1.0), harvested and fragmented by treating the cells to a 1min sonication in a water bath and pelleting the cells by centrifugation at 10krpm for 10min. (repeat 3x in 25ml of OT)
2) the final pellet was resuspended in 30ml of 0.1M $CaCl_2$ and 10mM TrisHCl pH8.0. This suspension was aliquoted 5ml each into a 15ml Falcon tube and pelleted in a clinical centrifuge at maximum speed, 10min.
3) the pellets were resuspended in 5ml of OT with 0,50,100, 150, 200 and 250 mM CaCl2 with 10mM Tris 8.0 and incubated on ice for 1hr
4) the treated cells were then poured into labeled petri dishes and exposed to U.V. light, 30W at 20cm for 10sec. A 1ml aliquote was taken and added to an eppendorf tube containing 1ug of vector DNA (eg pPUC29).The U.V.

treatment is repeated until the sample is used up for a final sample treatment of 40sec.

5) the treated cells and controls were then plated on OT plates with either KAN or CAM antibiotic and kept in the dark for 12hrs before returning to the 16/8 light/dark regiment.

6) plates are scored after 10-20 days

Modifications of method

1) cells were first separated into buoyant (active growing) and nonbuoyant (inactive) by initial centrifugation at 1/2 speed in a clinical centrifuge for 5min.

2) the floating cells were decanted and sonicated with probe sonication for 10sec and pelleted

3) the cells were washed in 10ml of OT (2x), divided in half, pelleted and resuspended in 5ml of either 150mM $CaCl_2$ or BG11 media.

4) proceed with U.V. treatment as above

5) survival is enhanced by either plating cells onto Nucleopore discs on 0.1ug/ml plates and transferring to full CAM plates after 24hrs or by plating on minimal antibiotic plates directly and adding full antibiotic after 24hrs by injecting an appropriate solution under the agar of the plate.

6) after the addition of vector DNA the cells were made to

30% in PEG-4000, heat shocked at 37 C for 5min and returned to ice before plating.

7) washes with OT were done with media minus bicarbonate.

8) minipreps of a pACYC184;2342 library were used as vector in this and all other procedures

II Transformation in BG11 (physiologic)

1) *S.maxima* grown to OD600=0.4 in OT (500ml) were harvested by sonication,centrifugation at 2.5kg and resuspended in BG11. This was repeated until filament size averaged less than 10 cells by inspection. The cells were then washed in same until the supernatent was clear.

2) The pellet was then resuspended in 30ml of BG11 and 200ul aliquotes added to minipreps of the pACYC184;2342 library, incubated room temp. for 30min and plated as described above.

NOTE this method was preformed on *S.platensis* UTEX 2340, *S.maxima* UTEX 2342, *Agmenellum quadruplicatum* UTEX 2268, and *Anacystis nidulans* UTEX 2434. Also using the vector pPUC29 that was not modified or was methylated with Hae III methylase.

III Transformation of permeaplasts

ref. Daniell etal

1) Cells grown, harvested and fragmented as described above resuspending each time in 50mM HEPES pH7.3

2) the pellet is resuspended in 50mM HEPES 7.3
1mM EDTA
2mg/ml lysozyme
shake in erlynmeyer for 1hr at 37 C at 60rpm

3) dilute 10x in 50mM HEPES 7.3 (ice cold) and pellet at 2.5kg, 10min,0 C.

4) resuspend in BG11 or OT(noHCO3) and add to vector DNA and proceed as previously described.

IV Transformation of Fe starved cells.

This method was developed out of discussions during the "Molecular Biology of Cyanobacteria Workshop" in St. Louis, MO. during the summer of 1987. The idea stemmed from some results presented on starvation induced proteins and the possible effect on cell membranes as a factor in competency.

Methods II and III were repeated using cultures of UTEX 2340 and 2342 that were grown in media without Fe added. The starvation time was varied from a few hours to several weeks.

DNA ISOLATION

I ref. Tiboni (1984)

This method was used to isolate DNA from a wild type *Spirulina platensis* for cloning into pBR322.

1) Harvested cells from a 500ml culture are suspended in 20ml of buffer containing;

Tris-HCl pH 8.3	50 mM
NaCl	50 mM
EDTA	0.1 M

to which is added lysozyme to 2 mg/ml and incubated, 37 C.

2) after 10-30 min with gentle stirring, SDS is added to a final concentration of 1% wt/vol. and the suspension kept at 0 C for 30 min.
3) to the mixture is then added 50 ug of proteinase K (or pronase) and incubated at 50 C for 30 min.
4) Sodium perchlorate is the added to 0.5 M and the suspension extracted with equal volumes of phenol,phenol/chloroform and finally chloroform with centrifugation in between steps to separate the phases.
5) DNA is then precipitated from the aqueous phase by the addition of sodium acetate to 0.2 M and 2 volumes of cold ethanol.

6) The DNA was pelleted by centrifugation at 10,000 g for 30min and the pellet resuspended in 0.1X SSC and the deproteinization repeated as necessary.
7) The resuspended DNA is then treated with RNase A at 50 ug/ml for 10 min at 80 C.
8) The solution is again deproteinized by extraction and the DNA precipitated with ethanol.

Modifications of method

a) It was found that the extraction of the lysate with phenol was extremely difficult and mixing of phases nearly impossible in our hands. We therefore chose to use CsCl-ETBr density gradients to purify the DNA either after extraction or with the crude lysate.
b) after the digestion with pronase, RNase is added and incubated as described, followed by the addition of sodium perchlorate.
c) This suspension resulted in a volume of approximately 23 mls to which is added CsCl and ethidium bromide to 40 ug/ml to a final density of 1.55 g/ml . The solution is the centrifuged at 44,000 rpm for 48 hrs at 18 C.
d) the resulting genomic band is removed from the tube with a syringe through the wall while visualizing under U.V. light. The aliquot is extracted with isoamyl alcohol until clear and desalted on Centricon 10 cutoff filters. The DNA is now ready for digestion.

II ref.B. Witholt (1976)

1) a 10ml culture of cells are washed in growth media and pelleted.
2) pellet is resuspended in 100ul of

Tris-HCl pH 8	200 mM
EDTA	0.5 M
sucrose	0.1 M
lysozyme	4 mg/ml

incubate room temp. 30 min (to 2 hrs)

3) lyse by osmotic shock by adding 200 ul double distilled water, let stand 2 min. Centrifuge (eppendorf) 5 min.
4) remove supernate by pipet and extract with equal volume phenol-chloroform.
5) precipitate DNA with ethanol

III ref S.J.Robinson (1982)

This procedure was developed for the preparation of spheroplasts for photosynthetic studies but was used in this lab to lyse cells for DNA isolations and as a procedure to prepare cells for transformation.
The following solutions are referred to in the procedure;

RB =	PEG-4000	7.5%
	CaCl	1 mM
	Hepes-NaOH	20 mM pH 7.5
EH =	EDTA	10 mM
	Hepes-NaOH	10 mM pH 7.5
	PEG-4000	5%
KE =	KCl	1.5 M
	EDTA	5 mM
	Hepes-NaOH	10 mM pH 7.5
LB =	PEG-4000	10%
	Tris	10 mM pH 8.0
	$MgCl_2$	2 mM
	$CaCl_2$	2 mM
	NaCl	40 mM
	lysozyme	0.01%

1) A 500 ml culture is grown to OD-560 = 0.5 and harvested by filtration
2) resuspend cells in 60 ml. of RB, sonicate 10 sec with

probe at 50% power, centrifuge at 3000 g, 5 min.

3) resuspend pellet in 60 ml EH, pellet
4) resuspend pellet in 60 ml EH, sonicate, pellet
5) resuspend in 60 ml KE, stir 5 min at 4 C.pellet
6) resuspend in 10 ml RB, pellet
7) resuspend in 50 ml LB, incubate at 37 C for 45-90 min.
8) visually inspect for spheroplast formation

Modifications of method for cell lysis

a) to the final prep in LB is added 2 volumes of distilled water to osmotic lyse OR the cells were pelleted and resuspended in 20% SDS,0.2M NaOH, incubated 5min at 4 C.
b) add 15 ml of acetate solution and incubate on ice 5 min.
c) the lysate is centrifuged 10K rpm 40 min, 4C.
d) the supernate is saved for CsCl density gradient

IV Potts M. (April 1984)

This method was modified from the literature for the isolation of chromosomal and plasmid DNA and RNA.

1) A 500 ml culture was harvested by filtration and washed in 30 ml TSED buffer 2X, pellet at 3kg, 5 min., 4 C.

TSED = sucrose 25%
EDTA 5 mM
Tris 50 mM pH=8.0

2) the pellet is resuspended in 80 ml STET buffer with 5mg/ml lysozyme and divided into 4 fractions, incubate 1 hr,37 C.

STET =	sucrose	8%
	Triton X-100	5%
	EDTA	50 mM
	Tris	10 mM pH 8

3a) to 2 aliquotes were added 6.0 ml of 5M NaCl, mixed and incubated 2hrs, 4 C (plasmid prep).

3b) the suspension was centrifuged at 10K rpm, 4C, 1hr. The supernate was poured into a fresh tube and treated with 50 ug/ml RNase A, 65 C, 30 min.

3c) the DNA was the isolated by CsCl-EtBr density gradient centrifugation.

4a) to the remaining fractions were added 1.0 ml 2M NaClO4, mixed and extracted with phenol preheated to 60 C, 2X. The phases were separated by centrifugation (RNA prep).

4b) the aqueous phase was precipitated with 2 volumes ethanol after adjusting the solution to 0.1M KCl, stored at -70 C.

4c) the suspension was centrifuged at 10K rpm, 1hr, 0 C and the pellet resuspended in 100 ul TAE (1X)

PLASMID ISOLATION

I ref. Simon, (1978)

This method used to isolate plasmids from the cyanobacteria *Phormidium luridum* (2.1 and 9.0 Mdal) and *Plectonema boryanum* (9.4 Mdal)

1) from a single colony, inoculate 10ml rich media and incubate over night
2) Inoculate 2.5ml of over night culture into a 2l flask containing 500ml of minimal media with antibiotic. Grow to an O.D.600 = 0.4-0.5
3) add 2.5ml of chloramphenicol (34mg/ml in ethanol) incubate 12-16 hours
4) harvest by centrifugation at 4000g, 10min, 4 C
5) wash in 100ml STE ice cold

STE = NaCl 0.1M
Tris-Cl pH 7.8 10mM
EDTA 1mM

6) resuspend in 10ml ice cold 10% sucrose, 50mM Tris-Cl pH8.0
7) add 2ml fresh lysozyme solution, 10mg in 0.25M Tris-Cl pH8.0
8) add 8ml 0.25M EDTA. Mix by inverting, place on ice for 10min.
9) add 4ml 10% SDS, mix quickly but gently

10) immediately add 6ml of 5M NaCl, mix gently and place on ice for at least 1hr

11) centrifuge 30min at 30,000rpm, 4 C. Pour off supernate and discard pellet

12) extract supernatent with equal volumes of phenol -phenol/chloroform-chloroform

13) ethanol precipitate

14) purify by CsCl-EtBr gradient

II Mandel,M. Personal communication

This method is used to isolate and purify plasmids from E.coli strains.

1) Pelleted cells from a 500ml culture, are resuspended in 17 ml of 25% sucrose, 50 mM Tris-HCl, pH 8

2) add lysozyme, 1 ml of a 10 mg/ml stock in 50 mM Tris-HCl pH 8

3) mix and incubate 30 min. at 0 C

4) add EDTA 2ml of a 0.5 M stock, mix

5) add SDS 2.5 ml of a 10% stock, mix

6) add NaCl 5.0 ml of a 5M stock, mix

7) keep at 0 C at least 3 hrs. or over night

8) centrifuge at 20,000 g at 0 C for 30min to 1 hr.

9) pour off supernatent, discard pellet

10) to supernate add CsCl (approx 0.92 g per ml. of super.) and ethidium bromide 40 ul/ml super (10mg/ml stock)

11) centrifuge at 44K rpm, 16 C, 40 hrs

12) visualize with U.V. light and remove the upper band with a syringe.

13) extract ethidium bromide with isoamyl alcohol and desalt by dialysis against TE buffer (or by Centracon-10 cutoff filter).

Modification of method

a) the cleared lysate is precipitated with 2 volumes of ethanol, pelleted at 10k rpm for 30 min and resuspended in TE.

b) the suspension is loaded onto a preparative agarose gel with loading buffer and electrophoresed at 60V for 2 hrs.

c) the plasmid band is visualized under U.V. and cut from the gel with a sterile knife.

d) the removed gel is loaded into a dialysis bag with a small volume of buffer, placed in the electrophoresis chamber and electroelute at 100V for 15 min.

e) after a short 2-5 min. reversed pulse the solution is removed from the bag and is ready for transformation, digestion etc.

f) this method has been used successfully for isolating DNA that has been previously digested, either for fragment removal or sizing of genomic DNA digests.

III ref. Lambert (1982)

This procedure was used for the small scale preparation of plasmids from cyanobacteria.

1) A 100 ml culture of stationary phase cells were washed 2X in growth media and pelleted
2) the pellet is resuspended in 0.5 ml of SET

SET = sucrose 25%
EDTA 0.1 M
Tris 0.05 M pH 8
lysozyme 5 mg/ml

incubate 1 hr 37 C.

3) add 75 ul of 10% SDS, mix, incubate 1 hr 37 C
4) add 150 ul 5M NaCl, mix by inverting, incubate at least 2 hr on ice.
5) centrifuge (eppendorf) 15 min room temp.
6) extract supernate with phenol-chloroform and precipitate DNA with ethanol.

Modification of method;

A scale up of this procedure was preformed at 10X the literature values and NaCl was replaced with $NaClO_4$ to a final concentration of 0.5M. The suspension was too viscous to work with and was therefore diluted 2X with TAE before extracting with phenol-phenol/chloroform-chloroform. The aqueous phase was precipitated with 2 volumes of

ethanol.The pellet is resuspended in TE and the DNA purified on CsCl-EtBr equilibrium gradient.

IV ref Wang (1988)

1) grow 2ml over night culture
2) pellet cells in a 1.5ml microcentrifuge tube
3) resuspend sells in 0.21ml of STET

STET =	glucose	8%
	Triton-X 100	0.5%
	EDTA	50mM
	Tris pH 8	10mM

4) add 15ul fresh lysozyme (10mg/ml) and vortex to mix
5) place in boiling water bath for 40-50sec
6) centrifuge immediately for 6min
7) transfer supernate to new tube and precipitate DNA with 1/10 vol 3M NaOAc and 1 vol isopropanol,-70 C 5min
8) wash pellet with 70% ethanol and vacuum dry

V ref. Engwall, (1985)

This method was designed for the identification of plasmid bearing bacteria by an in gel lysis.

1. 10^8 cells are resuspended in 500ul of TBE with 1% sarkosyl

TBE = Tris 89mM

Boric acid 89mM

EDTA 2.5mM

2.centrifuge 5min eppendorf

3.resuspend in 50ul TE, centrifuge 5min

4.resuspend in 25ul TBE with 10mg/ml lysozyme and 10% Ficoll, incubate 37 C 1hr.

5.add 5mg/ml pronase incubate 37 C 10min

6.load into well of 0.7% gel

7.layer on top 25 ul of SFB

SFB = SDS 10%

Ficoll 10%

bromphenol blue 0.05%

8. mix gently with pipet (3 strokes) and run the gel at 100 volts for 1-3 hrs

9. visualize with EtBr.

NOTE: This procedure apparently needs to be done on a vertical gel apparatus at relatively low current in order for the SDS/Ficoll overlayer to migrate through the treated cells and complete the lysis.

RNA ISOLATION (FROM PHOTOADAPTED CELLS)

ref. Chomczynski, (1987)

1) log phase cultures were diluted to OD600=0.1 in 100ml fresh O.T medium and exposed to differing light conditions;

   a. samples were taken during various times of the light/dark cycle (14hrs/10hrs)

   b. cultures were grown under green filters (>550nm) and red filters (<550nm) for 16hrs continuous.

2) 30ml aliquotes were taken, sonicated 3sec., pelleted at 2.5kg, washed with fresh media and repelleted and stored at -70 C

3) the frozen pellet was resuspended in 0.5ml of solution D

solution D =	guanidinium thiocyanate	4 M
	sodium citrate	2 mM
	sarkosyl	0.5%
	2-mercaptoethanol	0.1 mM
	pH	7.0

4) to this suspension is added sequentially

50ul	2M sodium acetate pH 4
0.5 ml	phenol
100 ul	chloroform/isoamyl 49:1

vortex between each step and finally incubated for 15min on ice

5) The mixture is then centrifuged at 10,000g, 20min, 4 C

6) The aqueous top layer is removed and precipitated with an equal volume of isopropanol and pelleted.
7) the pellet is resuspended in 0.3ml of solution D and precipitated with 2 volumes of ethanol.
8) the pellet is washed with 70% ethanol, pelleted and taken up in 20ul of RNAse free H2O.
9) 3-10 ug of RNA is denatured for 1hr at 55 C in glyoxyl and run on a 1.5% agarose gel in phosphate buffer with recirculation.
10) transfer to nitrocellulose is the same as described for DNA.

DNA COMPOSITION ANALYSIS BY HPLC

ref Padhy (1988)

DNA was isolated and purified by the method of Tiboni. Extractions and precipitation of DNA was repeated until the OD260/280 ratio equaled 1.8. Aliquotes of 300 to 450ug were hydrolysed under nitrogen at 180 C for 30min in 88% formic acid. The digest was then lyophilized and resuspended in100ul of elution buffer (50mM $NH_4H_2PO_4$ pH=3.3). HPLC was run on a Phenomenex "Spherex" 10 SCX column (250x4.6mm) at a flow rate of 1ml/min. Elution products were monitored by UV at 270nm. The strains analyzed were; *Anacystis* ATCC 27344, *Anabaena* ATCC 27892, *Pseudanabaena* ATCC 29344, *S.maxima* UTEX 2342

ESTIMATION OF Tm FOR *Spirulina sp.*

ref. Marmur (1962)

Purified DNA extracts (OD260/280=1.8) were desalted on Centricon-10 cutoff filters and resuspended in 1X SSC at a concentration of approximately 0.5 OD260. Thermal denaturation and reannealing were monitored by a Cary219 UV/vis spectrophotometer with the thermocouple attachment connected to the strip chart recorder. Temperature was controlled by using a jacketed sample cell attached to a manually controlled circulating temperature control water bath. The Tm values were estimated by hand from the inflection point of the temperature versus OD260 plot off the strip chart. The %GC value was calculated from the equation;

$$Tm = 69.3 + 0.41(G+C)\%$$

after normaization of the data to a Salmon sperm DNA standard.

BLOTTING AND HYBRIDIZATIONS

Blotting DNA to Filter Media

Two blotting methods were used throughout this work. The method of choice was Southern blotting to nitrocellulose (or nylon) from denatured gels using 20X SSC as described in Maniatis (see also Schieler and Schuell

manual for nucleic acid blotting to filter media). The other method used was electroblotting as described by BioRad. This method is used for nylon membranes which we found in general, not conducive to heterogolous probing due to the "blocking" requirements for this particular media effecting "stringency".

Blotting of DNA to filters

I ref. Maniatis

a) from gel to nitrocellulose

1) after running the gel and photographing it, soak the gel in several volumes of 0.25N HCl for 5-10min then 0.5N NaOH, 1.5M NaCl for 1hr/150cm^2 of gel, at room temp.
2) neutralize the gel by soaking in several volumes of 1M Tris pH 8.0 and 1.5M NaCl for 1hr/150cm^2, room temp
3) wet a piece of nitrocellulose cut to the exact size of the gel, in 2X SSC (prewetting in DD H_2O may be necessary).
4) assemble the capillary apparatus from bottom to top; 3MM wick, gel, nitrocellulose, 3MM paper, paper towels 6-8in thick, 500g weight
5) the reservoir should be full and the wick soaked with 20X SSC before assembly.
6) allow the transfer to run 8-16 hours, replacing towels as necessary.
7) disassemble the apparatus, peel off the filter

and rinse it in 6X SSC

8) blot the filter dry and bake it at 80 C under vacuum for 2hrs.

b) insitu binding of DNA from plates to nitrocellulose (or nylon)

1) place a labeled, sterile circle of nitrocellulose on to the surface of a plate containing colonies to be screened. Allow the filter to become wet.

2) mark the filter and plate to give positive orientation

3) remove the filter with forceps and place it on a puddle (0.75ml) of 0.5M NaOH, colony side up.Leave for 2-3min

4) blot filter dry and repeat once.

5) blot filter dry and place on a puddle of 1M Tris pH=7.4, 2-3 min

6) blot dry and repeat once.

7) place filter on a puddle of 1.5M NaCl, 0.5M Tris pH7.4. after 5min blot dry and bake 80 C, 2hrs under vacuum.

II electroblotting from gel to 'Zetaprobe"

ref. BioRad bulletin 1232 for electroblotting of DNA to 'Zetaprobe'. K.C.Reed

1) soak a piece of 'Zetaprobe' nylon membrane in distilled water for several minutes

2) Assemble the blotting sandwich;sponge, 3MM paper(3X), gel, Zetaprobe, 3MM paper (3X), sponge, in the holder and

place the assembly into the transfer chamber, with the membrane on the anode side

3) Fill the chamber with TAE (1X) and run the transfer at 60 volts for 30min to 4hrs with the cooling coil.

4) disassemble the apparatus, remove the membrane and place it on a pad of 3MM paper soaked in 0.4M NaOH for 10min.

5) rinse filter in 2X SSC and blot dry.

6) the filter is ready for use or may be stored after drying by baking at 80 C under vacuum.

Labeling of Probe DNA

All labelling was done with purchased kits as described by the manufacturer. Nick translation kits were purchased from Worthington Labs and BRL and were used throughout the work for labeling of plasmid DNA and restriction fragments.

In all cases the labeled probe was removed from unincorporated label by using minispin columns (Sephadex G-50) from Worthington. An alternate method of purification for all types of DNA (probe DNA, minipreps, etc.) was to use the NEN NENsorb-20 purification columns as described by the manufacturer.

Synthetic oligonucleotides were end labeled using DNA kinase (NEB) and gamma 32-P-ATP. The oligonucleotides used were;

RCP1121B manufactured by Richard C. Pendelton of BioSearch Inc (4/8/86) = GCT GCT TG(TC) CTC CGT GA(TC) ATG This sequence was derived from published amino acid data for a conserved region around the first cystine binding region in the beta subunit for CPC

B-N (beta-N) manufactured by Niel Riemer of U.H. Biotech Group (6/2/88) = ATG TT(TC) GA(TC) GC(TC) TT(TC) AC This sequence was derived from N-terminal sequence data from the beta subunit of CPC from *Spirulina platensis* also from N.Reimer

Hybridization

Dozens of hybridization protocols were tried in an attempt to hybridize genomic digests of *Spirulina* to either oligonucleotide probes or heterogolous probes obtained from the clone pTP1. Only one procedure was found to be successful, that of Lemeaux and Grossman. For reference sake, all other procedures are given in the appendix and summarized in table form.

It should also be noted that this procedure does not work well with nylon membranes nor does it work with biotinylated probes. Modifications are possible but were not pursued.

1)prehybridize 10-30min 65 C in

NaCl	0.5M
NaH2PO4	0.1M
Tris	0.1M
EDTA	2mM
SDS	0.1%

2)hybridize in same 65 C 18hrs with probe DNA denatured by boiling 10min in 0.4M NaOH

3)wash 10min each (3X) room temp. in

PO4 buffer pH7.0	10mM
EDTA	2mM
SDS	0.1%

4)wash 30min room temp

PO4 buffer pH7.0	50mM
EDTA	2mM
SDS	0.1%

5)autoradiograph filter.

LIBRARY CONSTRUCTION

Ligations.

Ligation conditions were performed as directed by the ligase manufacture with some modifications drawn from the literature.

The vectors used were pBR322, pACYC184, pUC8 and pUC12.

All were obtained from local labs. A BamHI cut and dephosphorylated pBR322 was purchased from NEB.

I Digested genomic DNA was fractionated on a 5-40% sucrose gradient and the 10-20 kb fractions were selected for ligation. Genomic and vector DNA were mixed in a 2:1 ratio in ligation buffer:

Tris pH=7.6	50mM
MgCl2	10mM
DTT	10mM

The mixture was heated to 60 C for 15 min and then allowed to cool slowly (0.5-1 hr) to room temp. To this was added ATP to 1 mM and 0.5U of T4 ligase. The reaction was run for 1.5 hrs at room temperature and then stored at -20 C.

II ref Struhl K., (1985)

1) vector and insert DNA are digested with the appropriate enzyme(s) and run on low melting agarose (FMC"SeaPlaque")
2) the ethidium bromide visualized fragments are cut from the gel in as small a volume as possible (30-50 ul)
3) the gel slices containing the relevant DNA segments are melted at 70 C for 5-15min and the combined in the appropriate proportions to give a final volume of 10ul.
4) after equilibration of the molten gel slices to 37 C,

10ul of ice cold 2X buffer containing T4 ligase is added and mixed quickly and the mixture incubated at 15 C for 3-24hrs.

ligation buffer :

Tris-HCl(pH 7.6)	50mM
$MgCl_2$	10mM
ATP	1mM
dithiothreitol	1mM
T4 ligase	1U
vector/insert	= 3 (0.1pmol tot)

5)prior to transformation the ligation mixture is melted at 70 C and diluted by a factor of 10 to 50 in TCM buffer:

Tris pH7.5	10mM
$MgCl_2$	10mM
$CaCl_2$	10mM

III ref. King and Blakesley,(1986)

1)prepare the ligation reaction mixture as described in method II with the addition of 5% PEG 8000.

2)incubate at room temp for 4hrs. Store at -20 C until use

3)dilute the ligation mixture 3-5 fold before transformation.

Transformation

Transformation of *E.coli* to generate libraries or to produce plasmid/recombinants were preformed by the method of Mandel using CaCl shock on strain HB101.

1) grow over night culture of *E.coli* HB101 in LB media
2) inoculate 1ml of over night culture into 100ml LB
3) grow at 37 C until OD600=0.2
4) chill on ice for 10min
5) spin out cells for 10min at 2.5Kg (Sorvoll GSA 4.5krpm)
6) suspend cells in 100mM $CaCl_2$ at 0C, incubate 25min at 0 C
7) spin cells as above and suspend pellet in 500ul,100mM $CaCl_2$ at 0 C.
8) incubate 1hr at 0 C.
9) Add suitable amount of DNA (plasmid or ligation reaction)
   e.g. 20ul DNA (0.5-1ug)
   100ul $CaCl_2$ treated cells
   mix and keep at 0 C for 25 min
10) incubate 5min at 37 C
11) add 2 ml of LB and shake 37 C for 1hr
12) plate serial dilutions on selective agar

Modifications of method

It was found that competent cells could be stored for up to a year by placing the cells in glycerol at -70 C.

1) after step 6 the cells are resuspended in 7.5ml of 100mM CaCl2 and 50% glycerol. The cell suspension is aliquoted into 200ul fractions and immediately placed into -70 C freezer.
2) cells obtain maximum competency after 24hrs.
3) aliquotes can be removed as needed, thawed on ice and used directly for transformation.
4) to 100ul of competent cells in a 1.5ml microcentrifuge tube, is added 1-10ul of DNA containing no more than 40 nanograms of DNA.
5) the suspension is incubated 30min to 1hr on ice
6) the cells are then placed in a 47 C water bath for 45sec and the returned to ice for at least 2min
7) 0.9ml of SOB media are added and the cells are incubated 37 C for 1hr

SOB media =	bacto tryptone	20 g/l
	yeast extract	5 g/l
	NaCl	10 mM
	KCl	2.5mM
	MgCl2	10 mM
	MgSO4	10 mM

8) plate on selective agar.
9) transformation efficiencies should be $>10^6$ cfu/ug DNA. Stored competent cells usually gave $>10^7$ cfu/ug pBR322.

Library Check by Fast Miniprep

This method was routinely used for rapid identification and preparation of recombinant plasmid DNA. With practice, as many as 60 individual clones can be prepared at once. In the case of the library screening, which required the preparation of a Southern blot of plasmid preps, over 300 clones were prepared at once by inoculating each culture with five or more colonies. Excellent results were also obtained with a large scale version of this procedure for a 500ml amplified culture. In this case the volumes were increased 50X and the incubation times doubled. The supernatent obtained prior to ethanol precipitation was run on CsCl gradient as previously described. The only difficulty is that the preparation is virtually devoid of chromosomal DNA, and therefore only the plasmid band is visible. The isolate must be run on a gel to insure it's identity.

ref. S.L.Swanberg via M.Mandel personal commun. 1988

1) inoculate 2ml of selective media from a single colony and grow cells to saturation
2) spin cells 20sec, 1-1.5ml eppendorf, remove super. with pipet or pour off carefully

3) add 100ul of GTE buffer, vortex to resuspend

GTE = glucose 50 mM

Tris pH8 25 mM

EDTA 10 mM

4) incubate 5min room temp

5) add 200ul alkali-SDS, mix well by tapping with finger

alkali-SDS = NaOH 0.2N

SDS 1%

make fresh every day from pellets

6) incubate 5min on ice

7) add 150 ul acetate solution, mix well by vortex max speed 2sec.

acetate solution = add 5M acetic acid to 5M potassium acetate to pH 4.8

8) incubate 5min on ice

9) spin 1min eppendorf room temp

10) transfer super. to fresh tube

11) add 0.9ml ethanol

12) incubate 1min room temp

13) spin 5min eppendorf room temp., discard super

14) wash pellet with 0.9ml 70 % ethanol, spin 20 sec

15) dry pellet under vacuum

16) resuspend in 20ul H2O, use 2.5-5 ul for digest

17) treat with RNase before loading on gel (optional)

RESTRICTION DIGESTS

All restriction digests were done in buffers supplied by the manufacturer or those described in the materials section. All digestions were done at 37 C and 1 to 5 U/ug DNA, except for BstY1 which was done at 65 C.

SUBCLONING

Subcloning of fragments from positive recombinants was done by the same methods described for library construction using primarily the in-agar ligation method. Vectors used for subcloning and sequencing were; pUC8 and 12, M13mp18,pBR322 , pZF18U and pIBI25. all vectors except for pBR322 have multiple cloning sites.

SEQUENCING

Sequencing protocols for double stranded templates were followed as described by the manufacturer. Systems used were: Klenow fragment (NEB), "Sequenase" (USB), and Taq polymerase (Stratagene). The primers used were the -40 universal primer for subclones in M13mp18 and it's plasmid derivatives (USB). The pBR322 clones were partially sequenced using "Sequenase" and the EcoRI (cw) primer and the BamHI (cc) primer (NEB). In addition the synthetic

oligomers B-N and RCP1121B were tested as primers for sequencing using the "Sequenase" system.

RESULTS

CULTURE AND GROWTH EXPERIMENTS

Growth on Inorganic Media

Growth on various media was dependent upon the strain used. Attention was focused on strains UTEX 2340, *S. platensis* and UTEX 2342, *S. maxima* (also SP-19 and SM-20 respectively) that grow only in full strength O.T.. Other media such as BG11, ASN III, WHBM, and sterilized sea water did not support growth and no attempt was made to adapt the strains to these media. Other strains used for comparison and controls were either grown in BG11 (fresh water organisms) or ASN III (marine organisms) and were maintained on agar or liquid media. Strains that would not grow on these media were abandoned. All strains used have been maintained on their respective media for over four years.

Growth of *Spirulina* on Organic Supplemented Media

Although investigated by others (Takiguchi) it was of interest to test mixotrophic growth on these strains to determine the possibility of auxotrophic mutation.

Published results on growth effects of amino acids on *Spirulina* (Takaguchi) were repeated on UTEX 2340 and 2342 (data not shown). For virtually every amino acid tested

there was a depression in growth rate at 20 ug/ml and that valine had the most pronounced repression and that asparigine had no effect or slightly enhanced growth.

In addition several (12) carbohydrates were tested with essentially the same results. At 50 ug/ml all carbohydrates repressed growth as compared to the control with the most significant repression being with fructose, lactose and trehalose (data not shown).

We also attempted to generate auxotrophs using both UV and NMNG. Consistent with the published literature, no auxotrophs were obtained.

Growth in the presence of heavy metals

Heavy metal tolerance was evaluated by growth on gradient plates and interpolating tolerance levels as a linear gradient of 0 to maximum. The metals tested were those available by convenience ($PbAc_2$, $CrCl_2$, $Co(NO_3)_2$, $NiCl_2$) . The strains tested were those collected from Oceanic Institute. The following table summarizes the results;

TABLE 6

RESULTS OF METAL TOLERANCE TEST

(maximum values (ug/ml))

metal strain	Pb	Cr	Co	Ni
AP-4	0	380	120	100
AP-15	0	420	120	0
SP-3	(150?)	350	150	80
SP-19S (UTEX 1928)	400	500	150	80
SP-19 (UTEX 2340)	0	500	70	100
SM-20 (UTEX 2342)	0	500	70	100

Growth on antibiotic supplemented media

Antibiotic resistance was evaluated in the same manner as the metal tolerance tests. The following table summarizes the results;

TABLE 7

RESULTS OF ANTIBIOTIC TOLERANCE TEST

(maximum values (ug/ml))

antibiotic / strain	kan	amp	cam	penV
SP-3	100	0	0	0
SP-19 (UTEX 2340)	100	100	0	20
SP-19S (UTEX 1928)	100	0	0	25
SM-20 (UTEX 2342)	100	0	0	0
AP-4	100	0	0	30
AP-14	100	90	0	50+

RESTRICTION ENZYME ASSAY

The initial work done on this topic involved chromatography of total cell lysate and assay of the fractions for restriction activity on pBR322. The approach

was a compiled procedure of general protein purification methods. Our objective was to identify restriction activity by digestion patterns and use this information to select or construct an appropriate vector for transformation studies.The results obtained appeared as nonspecific DNAse activity in a broad peak off of the CM cellulose column whose maximum was at fraction 27 (out of 50) at a elution salt concentration of 0.3%. Due to the broad peak and nonspecific nature of the digestion analysis, we interpritated our results as poor separation and/or degradation of the enzyme(s).

Some time later we picked up the work again with a new, simplified procedure (Whitehead). This allowed us to run several controls on strains documented to have type II restriction enzymes as well as several test strains. The test DNA was pBR322. Our results are shown in fig.2 and summarized in table 8.

Fig.2

Restriction enzyme analysis of select cyanobacteria

by the method of Whitholt et.al.

lane 1, Synechococcus ATCC 27180 + pBR322

2, " no DNA

3, Plectonema ATCC 18200 + pBR322

4, " no DNA

5, Anabaena ATCC 27892 + pBR322

6, " no DNA

7, Pseudanabaena ATCC 29344 + pBR322

8, " no DNA

9, Anacystis ATCC 27344 + pBR322

10, " no DNA

11, Nostoc ATCC 27347 + pBR322

12, Spirulina subsalsa UTEX 1954 + pBR322

13, Spirulina maxima UTEX 2342 + pBR322

14, pBR322

15, lambda- Hind III marker

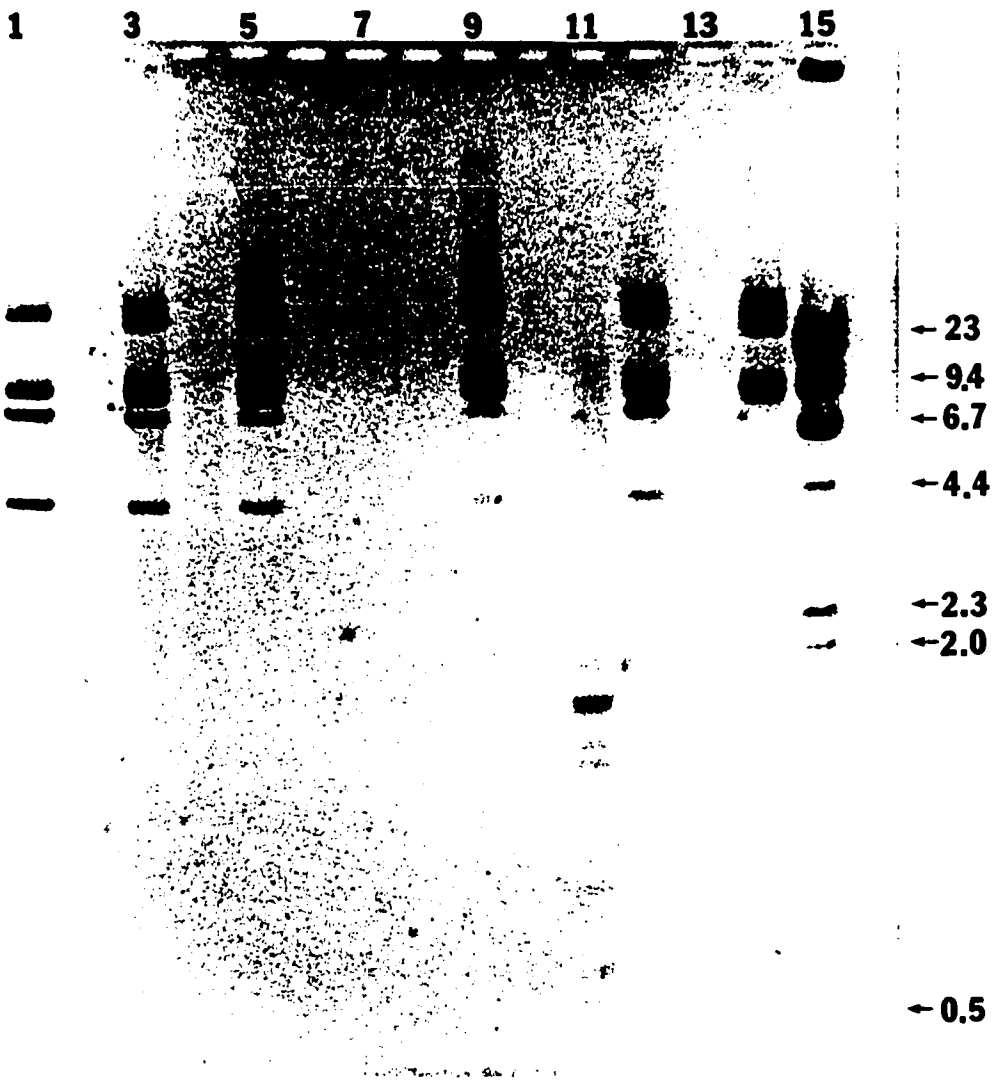

1
3
5
7
9
11
13
15
23
9.4
6.7
4.4
2.3
2.0
0.5

TABLE 8

RESULTS OF RAPID SCREENING FOR RESTRICTION ENZYMES

test strain	reaction conditions	effect
Synechoccus ATCC27180	'M' buffer, 1h,37 C	weak cutting discrete bands
Nostoc ATCC27347	" , " , "	complete cutting discrete bands
Plectonema ATCC18200	" ,2hrs, "	none
Anabaena ATCC27892	" , " , "	"
Anacystis ATCC27344	" , " , "	"
Spirulina subsalsa UTEX1954	" , " , "	"
Pseudanabaena ATCC29344	'M' buffer,30min,37 C	complete digestion,no bands
Spirulina maxima UTEX2342	" , " , "	"

(Thanks to De Fu (Howard) Chan for his assistance in obtaining this data and for the preparation of the DNA for HPLC assay)

The procedure used for this assay takes about 3hrs to run from cell pellet to final gel. The supernatant obtained is quite clear with an occasional sample containing a slight tint of biliprotein. We therefore decided to scale up the procedure as a starting point for column purification of the *Spirulina maxima* preparation. We hoped that we might be able to separate multiple restriction enzymes or remove the DNAse activity. What we obtained was very similar to our previous results in that a broad band of nonspecific DNAse activity was observed around the middle of the elution gradient at0.15 to 0.2M NaCl.

TRANSFORMATION OF SPIRULINA

In no case were treated cells induced to express vector markers except in the case of the physiologic transformation of *A.nidulans* 2434 with pPUC29 which was used as a control.

In some cases, in particular those involving Fe starved cells, tolerance to up to 3ug/ml chloramphenicol

could be developed over 30 or more days. However no plasmid or vector homologous genomic DNA, could be detected.

Nevertheless some observations on the survivability of the cells with regard to the various treatments are worth noting. First the cells are quite sensitive to osmotic lysis. Therefor the experiments involving calcium shock or spheroplast preparations, resulted in virtually total lysis of the cells upon plating. In addition, the calcium treatments resulted in an aggregation of the cells that were virtually impossible to resuspend. The highest survivability was observed with cells that were washed in BG11 media or OT media without the sodium bicarbonate.

The selection methods attempted, again based on survivability of plated cells, indicated that the under-agar injection of the antibiotic gives the best results. This no doubt has to do with the slow diffusion of the selection agent up through the agar giving the cells more time to express resistance. However a higher number of false positives are possible thus requiring proper antibiotic concentrations and several replatings of positive strains.

CONJUGATION

At the start I should admit that this method may be the most probable technique for successful transformation

of filamentous cells and was not well investigated. Although no transconjugants were obtained in the few experiments that was tried, there may well be modifications that might work. There are several variations that could be tried not the least of which is the use of different vectors. Pretreating the cells is another possibility which includes using starved cells or spheroplasts.

There does seem to be a compatibility problem between the standard growth media mixtures used in the conjugation procedure. The alkalinity and salinity of OT media is no doubt disruptive to *E.coli*. It has been demonstrated that even low concentrations of amino acid can induce mixotrophic lysis of *Spirulina*. Control samples plated on OT+0.5%LB usually lysed within a few hours after plating.

DNA AND RNA PREPARATIONS

It was found that *Spirulina* DNA was unusually difficult to isolate and digest. The exceptional amount of protein present and the rigid cell structure proved difficult to separate. The extended proteinase treatment was helpful, however even after repeated phenol and chloroform extractions, protein , pigment and polysaccharide contamination persisted. Purification on CsCl- ethidium bromide gradients was necessary.

Interestingly, this purification produced it's own set of artifacts. One or more bands would appear in the preparation at a higher density than the chromosomal band. Visual inspection and OD260 readings after extraction of the ethidium bromide indicated that the bands were either bilins if colored or polysaccharides if clear.

The approximate melting points of the DNA from the strains obtained from the UTEX collection were determined.

TABLE 9

APPROXIMATE* Tm OF SPIRULINA STRAINS

UTEX #	Tm obs	Tm calc	%GC
1318	85.8	81.9	30.7
1926	86.6	82.7	32.7
1928	86	82.1	31.2
1954	89.3	85.4	39.3
2340	93.5	89.6	49.5
2342	92	88.1	45.8

* normalized to salmon sperm DNA of Tm_{obs}=91.4 and Tm_{ref}=87.5

The first four strains listed are marine strains and were grown in ASNIII media. The last two strains are brackish strains and were grown in OT media.

DNA composition by HPLC

The intent of this analysis was to determine if there might be extensive modification of *Spirulina* DNA that might be involved with the difficulties in digestion and hybridization. The chromatograms for the strains tested are given in fig 3. Integration of the peaks and normalization to Salmon sperm DNA control were done. Quantization of nucleotide ratios were based on peak area percentage, for the four peaks, and the documented values for salmon sperm DNA (CRC Handbook of Biochemistry). The best results were obtained when only the thymidine and cytosine peaks were used.

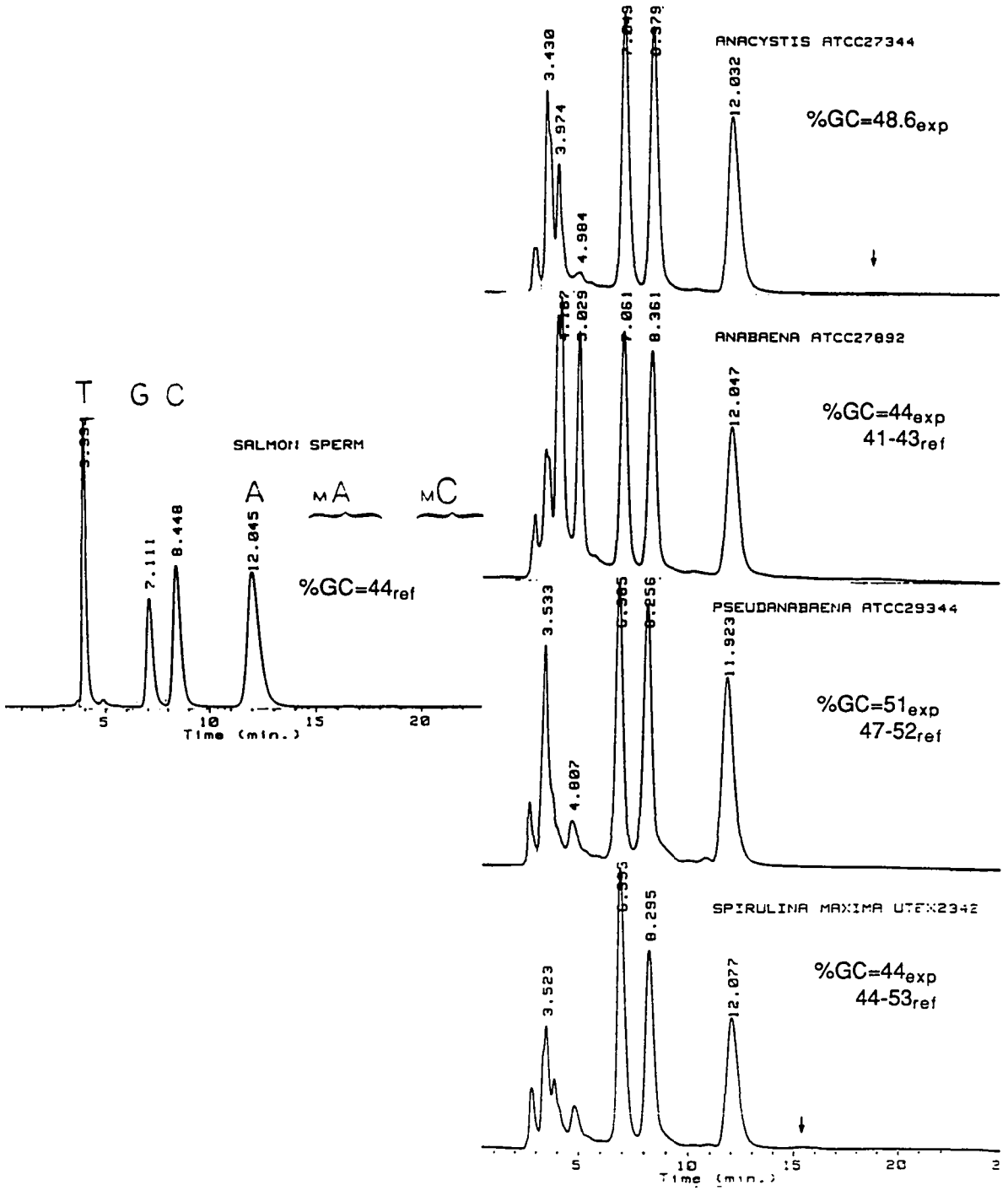
The values obtained compare reasonably well with our Tm values for Spirulina maxima UTEX2342. The results do not indicate any significant amount of modified DNA as expected by the literature (Padhy). There is a small peak appearing on an expanded view of the chromatogram corresponding to a methylated adenosine. A very crude estimate places the amount at <0.5%. There is a previous report on the observation of 5-dimethylaminopurine at 0.36% in *Spirulina* (Pakhomova).

The peaks surrounding the thymadine peak were unidentified artifacts also observed in the referance literature. UV spectra of these peaks show no

characteristic profiles outside of the 220-280nm range and are probably degraded bilins that copurified with the DNA.

Figure 3.

HPLC Chromatograms of DNA hydrolysates


T G C
SALMON SPERM
A mA mC
%GC=44ref
7.111
8.448
12.045
5
10
15
20
Time (min.)
ANACYSTIS ATCC27344
3.430
3.974
4.984
12.032
%GC=48.6exp
ANABAENA ATCC27892
3.029
7.061
8.361
12.047
%GC=44exp
41-43ref
PSEUDANABAENA ATCC29344
3.533
4.807
11.923
%GC=51exp
47-52ref
SPIRULINA MAXIMA UTEX2342
3.523
8.295
12.077
%GC=44exp
44-53ref
5
10
15
20
Time (min.)

RESULTS OF HYBRIDIZATIONS

Hybridizations using fragments from the C-PC clone pTP1 which was digested with HindIII and PstI, giving separate probes for the alpha and beta subunits, gave positive hybridizations (Fig.4) to genomic digests of *Spirulina maxima* UTEX 2342 as given in table 9. These enzymes were the only digests that gave distinct patterns. All other enzymes produced smears both on the gel and autoradiograph.

The banding pattern for ClaI was identical for both subunits indicating that the entire gene was contained within the fragment. The multiple bands appearing in the ClaI digest indicates that either a gene duplication exists in the genome or that the probe is hybridizing to both the C-PC gene (2.7kb) and the APC gene (5.5kb), the latter explanation being the most probable due to the fact that the intensity of the bands are quite different.

The pattern obtained for the PstI digest gave a single intense band for both subunits of nonidentical size, 4.1kb for the alpha subunit and 3.1kb for the beta. Once again faint banding appeared at a higher molecular weight (approximately 8kb) probably due to the APC gene.

There were no clear banding patterns obtained for any of the other restriction enzymes. This is due to the

ineffectiveness of most enzymes on Spirulina DNA which has been observed throughout the study and by others (Tiboni et.al 1983). However after passing cloned DNA once through *E.coli*, this problem no longer is present(except for the apparent lack of sites for most of the enzymes tested).

Other enzymes tested on genomic DNA are PvuI and Hpa I and II which also give inconclusive hybridization signals (Fig. 5.). However inspection of the digestion patterns in EtBr stained gels for Pvu I and Hpa II are interesting in that Pvu I appears not to digest the DNA at all compared to the control and that virtually no DNA is visible after Hpa II digestion.

Fig. 4

Autoradiographs of genomic blots hybridized to alpha and beta fragments of pTP1 under the conditions described by Lemeaux and Grossman.

a) alpha probe to digests of UTEX2342;

lane 1 BglII, 2 ClaI, 3 EcoRI, 4 PstI, 5 Bgl/Cla, 6 Bgl/Eco, 7 Bgl/Pst, 8 Cla/Eco, 9 Cla/Pst, 10 Eco/Pst

b) beta probe, same as a)

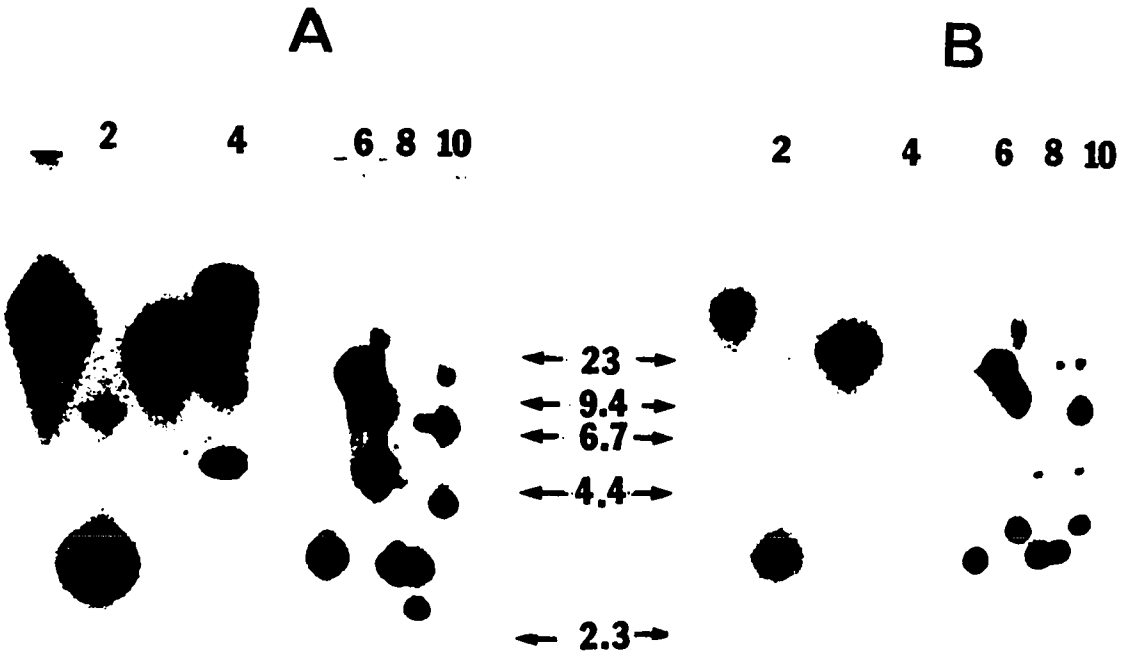

A
B
2
4
6
8
10
2
4
6
8
10
23
9.4
6.7
4.4
2.3

Fig. 5

Restriction digests of SP-19(UTEX 2340) genomic DNA

lane 1,marker DNA, 2,control DNA, 3,EcoRI, 4,BamHI, 5,PvuI, 6,HpaI, 7,HpaII, 8,HindIII, 9,Bam/Pvu, 10,HpaI/Hind, 11HpaII/Bam, 12,HpaI/HpaII, 13,Pvu/Hind, 15,marker DNA

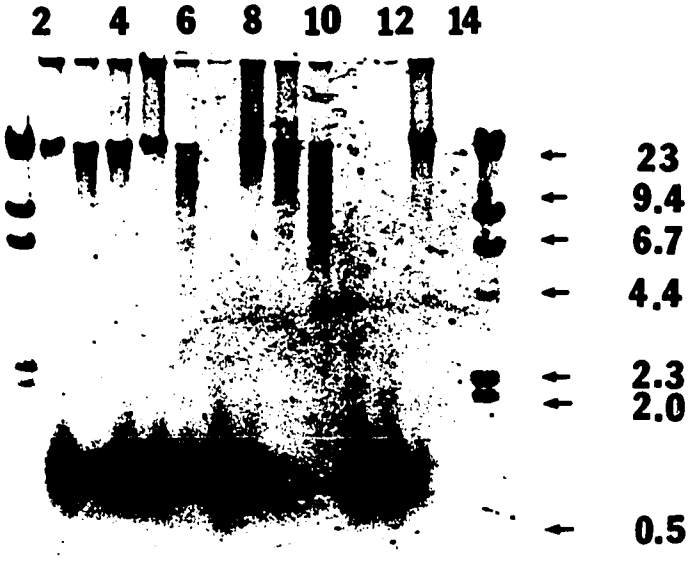

2
4
6
8
10
12
14
23
9.4
6.7
4.4
2.3
2.0
0.5

TABLE 10

GENOMIC RESTRICTION FRAGMENTS FOR C-PC

(HYBRIDIZING TO THE alpha AND beta FRAGMENTS OF pTP1)

(kb +/- 10%)

alpha

Bgl	Cla	Eco	Pst	Bgl/Cla	Bgl/Eco	Bgl/Pst	Cla/Pst	PstEco
35	3	22	8.4	3	12	8.4	7.1	7.1
			4.2			5	3	4.1
						4.2	2.5	

beta

35	3	22	3.5	3	12	8.4	12	8
					4.7	3.5	3	4.7
								3.5

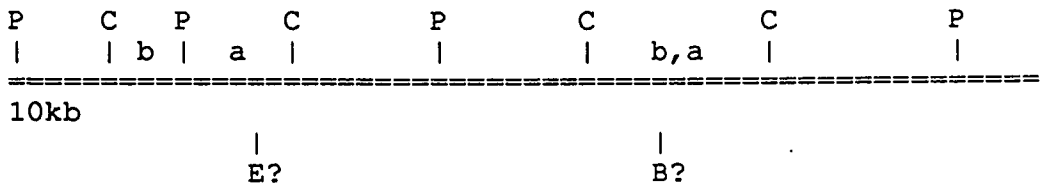
Cla/Eco (beta)

4.7

3

Fig. 6

Tentative map of *Spirulina maxima* 2342 C-PC genome



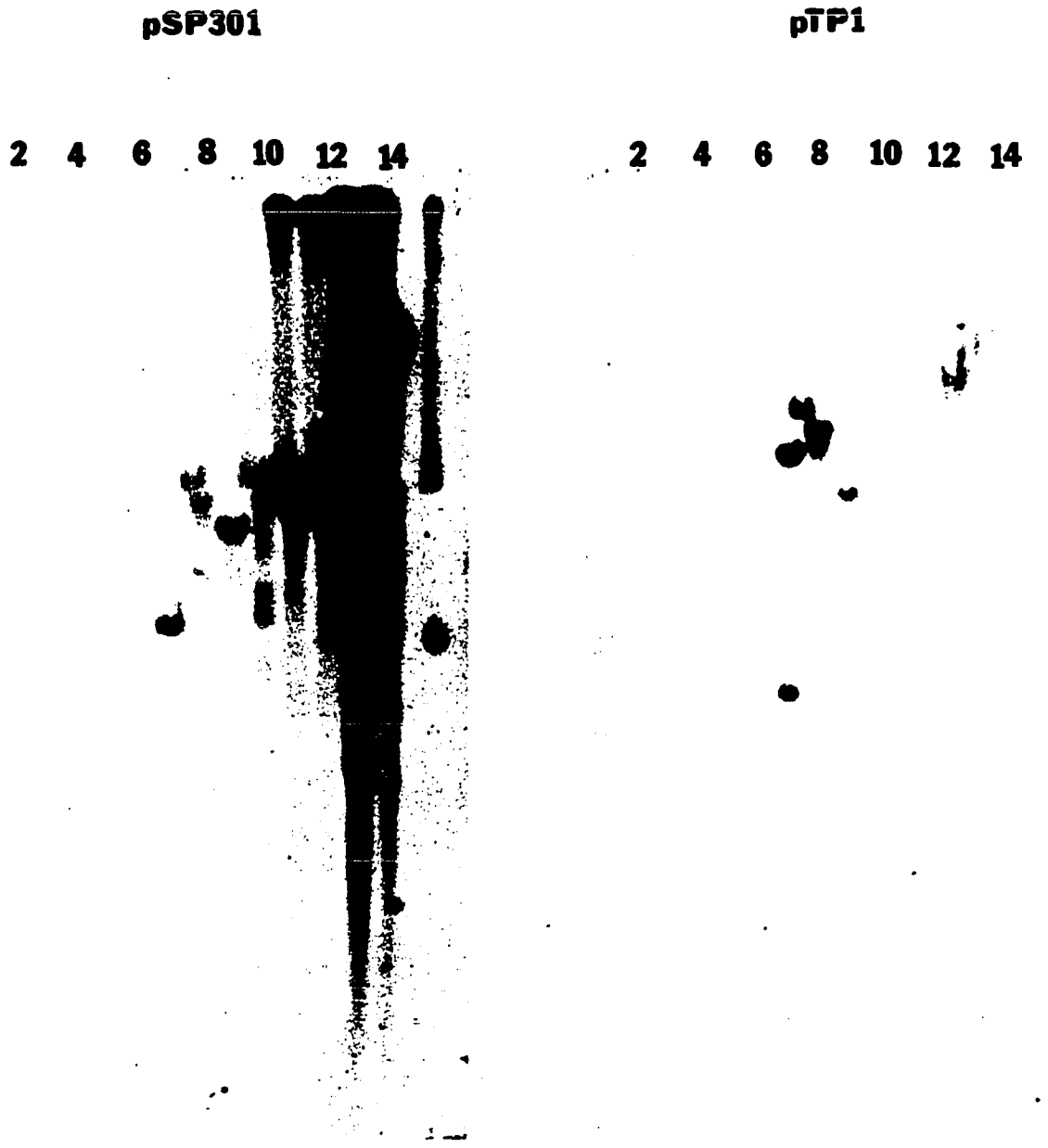
To evaluate the effectiveness of our hybridization techniques we managed to obtain the clone for the ribulose-bis-phosphate carboxylase gene from *Spirulina platensis* (Tiboni), pSP301. Hybridization under standard conditions as well as the method reported herein were successful (Fig.7). This would indicate that the hybridization problems are not so much with *Spirulina* DNA as a whole but rather with the C-PC gene specifically. The use of oligonucleotide probes for the phycocyanin gene were unsuccessful. The probe RCP1121b did pass control tests on the clone pTP1 but gave poor results on genomic digests and no results on *Spirulina*. The probe B-N never labeled to satisfaction (possibly due to incomplete deblocking of the oligo) and all hybridizations were negative.

Other probes used were clones pRMR2 (a nifHDK from *Rhizobium*), pTH228 (a methionine synthetase gene from *Anacystis*), and two standard cloning vectors used for their marker genes, pACYC184 (cam^r) and pUC-4K (kan^r), the former used to determine legitimate transformants and the later to look for the presence of an endogenous kanamycine resistance gene. Only pTH228 gave any kind of results although weak, the other probes producing no signals aside from an unexplainable artifact that occurred only in lanes that contained Cla1 digests.

Fig. 7

Comparative hybridizations using probes pSP301 and pTP1 under standard conditions (Tiboni,1983) to BglII and ClaI digests.

lanes 1&2,UTEX 552, 3&4,UTEX 1318, 5&6,UTEX 1954, 7&8,UTEX 2268, 9&10,UTEX 2434, 11&12,UTEX 2340, 13&14,UTEX 2342, 15,marker DNA


pSP301
pTP1
2 4 6 8 10 12 14
2 4 6 8 10 12 14

LIBRARY SCREENING

The colony lift hybridization for screening of all libraries resulted in little more than artifacts. Alleged positive colonies yielded either no plasmids or false positives upon secondary screening.

Upon the construction of the EcoR1/BglII 6-10kb partial library in pBR322, and screening of over 250 recombinants with no results, it was felt that once again some form of interference was preventing the detection of the C-PC clone. It had been determined that the Eco/Bgl fragment must be in the 10-20kb fraction, the library had been checked for proper sized inserts and replated to 100% recombinants. It was therefor decided that something must be interfering with the colony hybridization,probably cellular debris, being analogous to protein blocking agents.

Plates containing the same selected recombinants, arranged in a grid pattern were picked and inoculated 6-8 colonies per tube into 2ml of amp-LB and incubated overnight. Minipreps of these mixed cultures were run out on a 0.5% gel, blotted to nitrocellulose and hybridized with the C-PC alpha fragment. Groups containing positive signals were rescreened in the same manner until single colonies were located.

Several clones were isolated of different size or restriction pattern which were positive to alpha C-PC hybridization on southern blots of minipreps (Fig.8,9). These were designated pSMPC172,181,185 and 186 with insert sizes of approximately 8,10,12 and 6kb. After CsCl purification of these plasmids, digests were directly cloned or separated by electrophroesis on SeaPlaque agarose gels and select insert DNA was subcloned into the vector pBR322 or M13mp18 for further digestion and sequencing. Sequencing was done by primer extension and dideoxy termination as described by the manufacture of the sequencing kit (New England Biolabs and United States Biochemical).

TABLE 11

RESTRICTION FRAGMENTS HYBRIDIZING TO THE ALPHA FRAGMENT OF pTP1 (KB +/- 10%)

SMPC172

Cla/Eco	Cla/Pst	Cla/Ava	Ava/Eco	BstY1
6	5.5	9.5	3.2	1.4
				0.7

SMPC181

Cla	Eco	Cla/Eco	Cla/Pst	Pst/Eco	Hind/Pst	Hind/Eco
8.9	8.9	6.6,3.5	1.0	8.1	9.1	7.9

TABLE 11 (cont.)

Ava	Ava/Cla	Ava/Pst	Ava/Eco	BstY1
5.1	3.7	2.0	5.5	1.4
	2.1		2.9	0.7

SMPC185

Pst	Cla/Eco	Cla/Pst	Pst/Eco	Ava	Ava/Pst	Ava/Eco	BstY1
8.9	6	5.5	8.1	4.9	2.5	3.4	1.4
7.2			1.0			2.8	0.7

SMPC186

Cla	Eco	Pst	Cla/Eco	Pst/Eco	Hind/Pst	BstY1
6	6	4.5	6	4.5	4.5	1.4
		2		2	2	0.7

Fig. 8

Restriction digest of pSMPC clones

a) pSMPC172, lane 1 EcoRI, 2 Eco/Cla1, 3 Eco/PstI, 4 Eco/AvaI,
5 Eco/BstYI, 6 ClaI, 7 Cla/Pst, 8 Cla/Ava, 9 Cla/Bst,
10 PstI, 11 Pst/Ava, 12 Pst/Bst, 13 Ava, 14 Ava/Bst,
15 BstYI

b) pSMPC181, lanes 1-15, same as a)

c) pSMPC185, " , "

d) pSMPC186, " , "

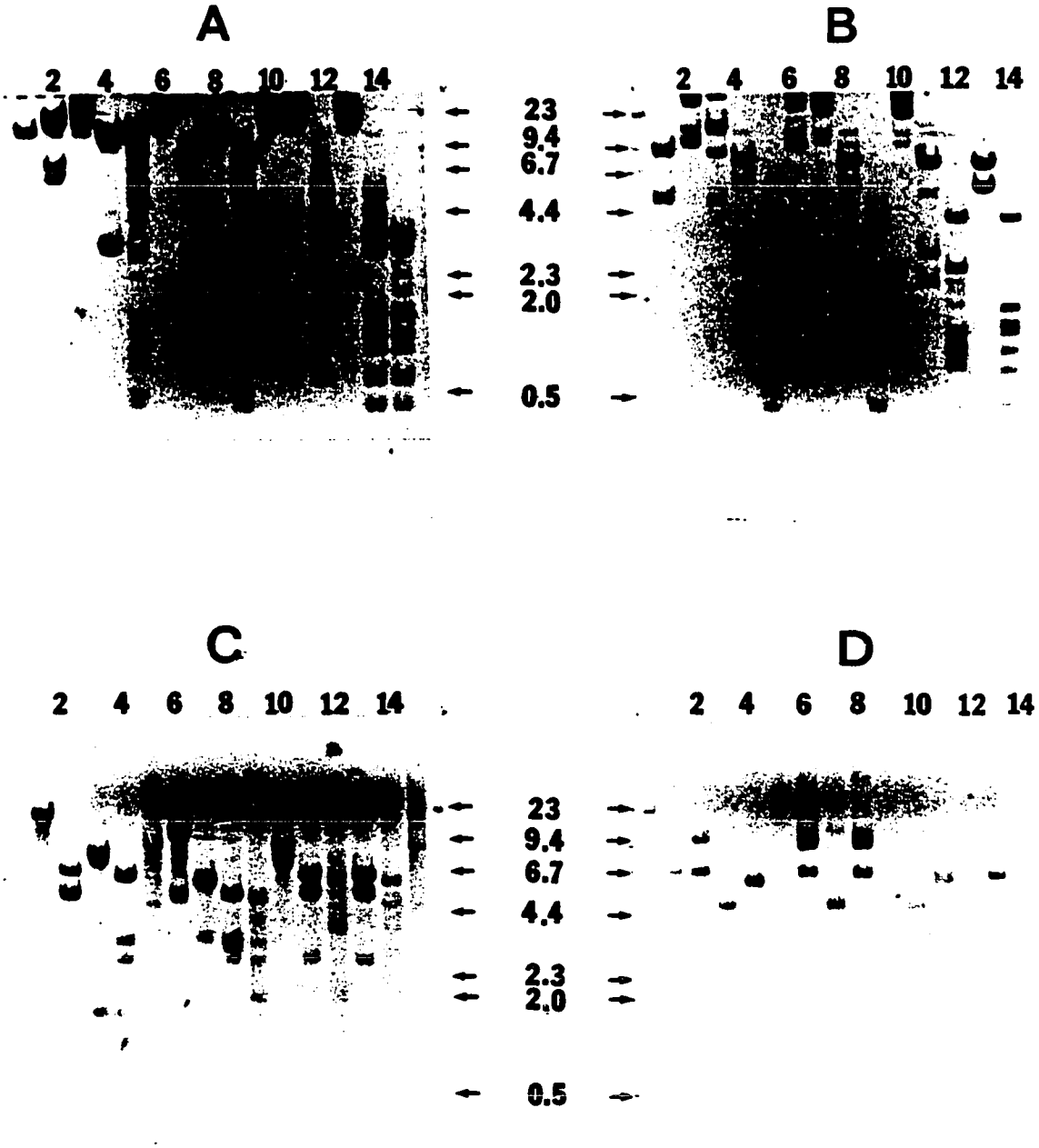
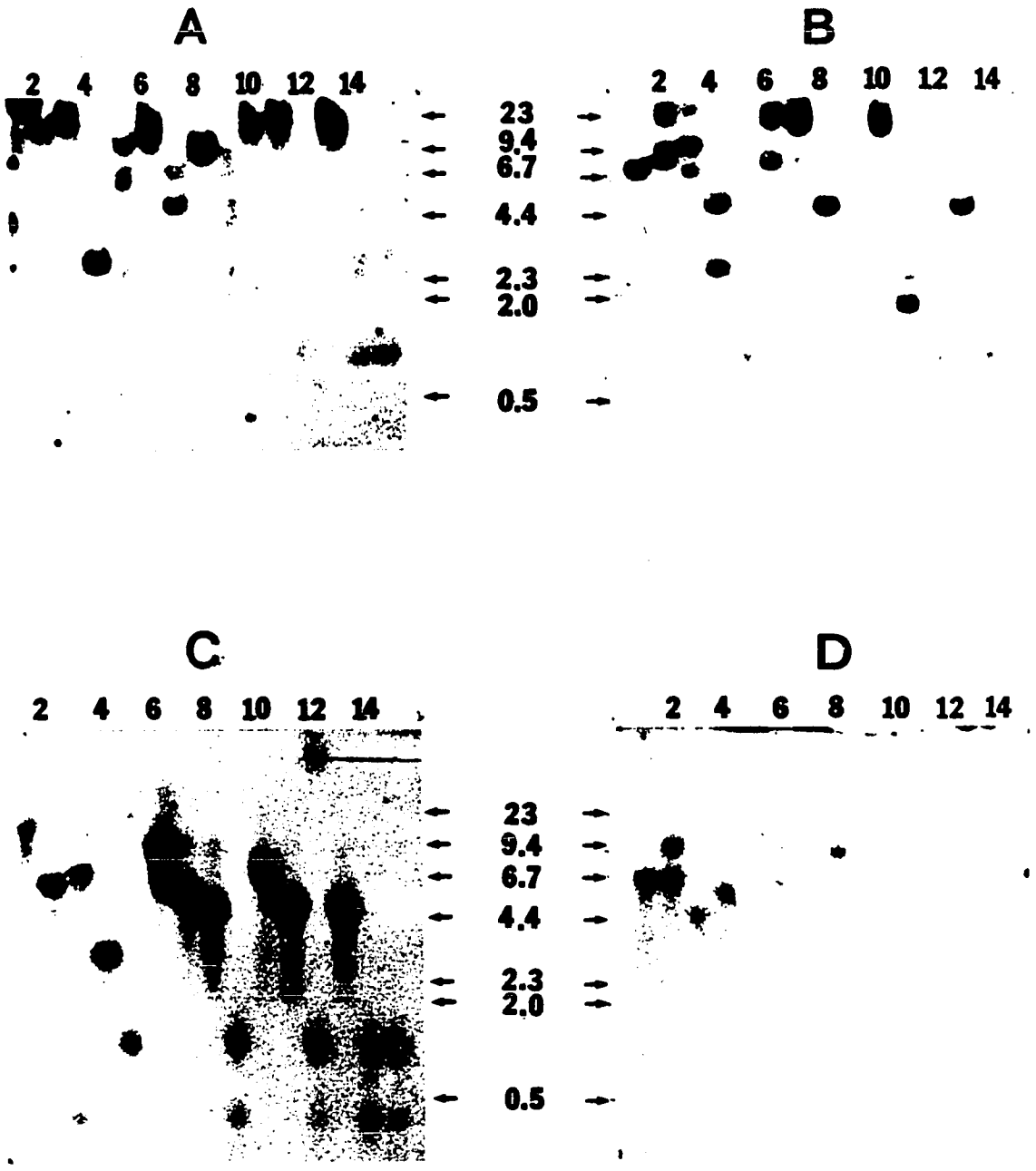

A
2 4 6 8 10 12 14
23
9.4
6.7
4.4
2.3
2.0
0.5
B
2 4 6 8 10 12 14
C
2 4 6 8 10 12 14
23
9.4
6.7
4.4
2.3
2.0
0.5
D
2 4 6 8 10 12 14

Fig. 9

Autoradiograph of hybridization with alpha fragment of pTP1 to blots of gels described in Fig. 8

a)pSMPC172, b)pSMPC181, c)pSMPC185, d)pSMPC186


A
B
C
D
2 4 6 8 10 12 14
23
9.4
6.7
4.4
2.3
2.0
0.5

These digests were based on enzymes useful for cloning into the polylinker found in the M13 series up to M13mp19. All enzymes and combinations not listed, did not cut the insert, only cut it once or produced spurious results.

As is apparent from the above table, the BstY1 digest gives the smallest usable fragment for subcloning of the structural region and gives virtually identical fragments for all clones. We pursued these fragments solely. Once again, the obvious route turns out to be the most difficult.

Subcloning into the Bam site of vectors pBR322, pUC12,pZF18U and pIBI25 proved unsuccessful. After much difficulty we managed to obtain from an M13mp18 subclone of pSMPC172 and 181, preparations that indicated inserts in both the RF and single stranded forms. The digests of the RF form to verify insert size however produced only the vector and a background smear. The experiment was repeated in a fresh cell line of JM103 with the same result. After two rounds of transfection the single strand for no longer indicated any insert present (It is noted by Dr Stiles that JM103 has a "P" restriction system that may be the problem).

RESULTS OF SEQUENCE ANALYSIS

The majority of the data obtained was from the "Sequenase" protocol. This system has, as do most sequencing systems, inherent errors. Compression around G-rich regions is well known and accounted for in the protocol with a dITP substitution for dGTP. This however was not a problem in this case. We did find that occasionally there would be bands that would run across all four lanes and usually corresponded to an adenosine base. On good sequencing runs such as with the Eco primer, this would happen no more than one or two times per hundred bases. On poor runs such as with the Bam primers, even when the gel was unreadable, these anomalies were more frequent.

The confidence level of the data, as determined by using the provided M13 control sample, was excellent. The control data in no case produced any erroneous data. The sequence data obtained was generally starting 60 to 100 bases from the primer and could be read to around 400 bases from the primer depending on the type of gel run.

pSMPC172

SMPC172ECO

TCCTTTGTGG AAGTAGTTTC TAGCTTTCGC AACTAGCTTC GCATCTGATG

TCATGCCCGT AACATTAACC AAATTATGTG GTCTAGTTTA ACGAAGGTCT

CAGATGAGAC GACTCTGAAT AGTAGTTGAC CATCCCTTAT GATTGGTTGT

CTGCTATCAG GCTGATGAGT GCTGTTATGT GTCTTACACT TACACTCT

SMPC172BAM

GGATT(A)GCGA TGTACAGAGC GAGCA(A)GTCG CGCTCAA

Data obtained from double strand Sequenase and Klenow sequencing of the intact clone does not show any significant homologies to any of the available published C-PC data. The EcoR1 end that was originally suspected to be within the structural gene gave limited homologies within short segments of the alpha c-terminus for the first 300 bases of the clone. A search of the EMBL gene data bank with the reverse complement of the given sequence, produced homologies to two biliprotein genes among the top 40 fits At number five there was the *Calothrix* cpeA and B genes and at number 27 there was the cpc genes. Using the "FASTN" program the homologies were initial=68, optimized =110 for cpe and 60 and 92 for cpc.

Using the D.P.S.A. program there are potential ORF regions for the reverse and reverse complement sequence of the Eco prime region. Potential in that any reading frame that produced two or less stop codons with the other reading frames showing numerous stops was considered a potential ORF.

Sequence data from the Bam primer end was extremely difficult to obtain for all clones except for pSMPC181. Again the data obtained as with the Eco primer, produced no significant homologies with the data base. There is no ORF for this sequence.

pSMPC181

SMPC181ECO

CTAACATCAA AAGGTCACTG AATAAGTTAA TCATGATTCACTTAGTTAAT
ACATCACTCA TGGCTCTAAG AATTGAGCCA ATAGCATTAAGACGACTTCA
CTTCCCAACC AGCTGAGATA TACCCACTAG CCCATACTCA TCATTAACGG
ACACTAGATA TGTATGCGTT CTACTGAATG TCACCTTGTC TACCTTGCAT
T

SMPC181BAM

ATTGCGCATT CCCGGTAATA AAGGAATTGC GGGTTATGTG GCTTCCAGAG
GTCAAGTGCT AAATATTACC AATGCTTATG CAGACCCTCG CTTTGACCCG
ACAACCGATC GCAAACTGGC TATCGCACTC GTAATATCTC TGTATGCCAG
TTGACACGCT AAAGGTGAGC TGATTGGTGT CACTCAGTTA TTATAACTAA
GGCGTTCACC GATCTCGTGAG GATTCT

Early hybridization data from the original clone indicated that the EcoR1 end of the insert was homologous to the alpha subunit of the probe DNA. The EcoR1 site appeared to lie somewhere in the middle of the structural

gene and sequence data should give the complementary strand, progressing from 3' to 5'. The clone pSMPC181 therefor must represent a genomic fragment containing a portion of the upstream alpha subunit, all of the intervening sequence, presumably containing linker peptide genes and at least a portion of the beta subunit of the down stream gene. However the sequence data obtained from the EcoR1 or BamH1 primer reaction does not seem to show any significant homology to any of the biliprotein sequences in the available data bases. Using the D.P.S.A. program one can find some homologies among the data in short regions using various selection parameters but the matching regions selected by the program do not compare.

There is an ORF for the given Eco primed sequence in the third reading frame. The Bam sequence has no ORFs.

pSMPC185

SMPC185ECO

GTTGGATCGT ACAGAAGTCT CGTCAGTCCA AAGTCTCGTC AGTCCTCCCA
TATCTAATTC CTTAAAGGAG TGCATGATGT CTTGATGCAG CTTTTCGGTC
GTCTGTGGAG TCTGTCTGAT CTATCATATT CGCCATTATT CTCTCTGTCG
GAAGTGCTGG AACACAGTCT AAGTCGTCTA CTATTACTAT GCGGGACTGC
AGTCTACACG ATAC

No homologous pattern has been found for the sequence data obtained from the Eco and Bam primers. It has been of particular difficulty to sequence clones 185 and 186. Both CsCl preps and minipreps of any preparation have been labile. For unexplainable reasons all preparations have degraded with storage, particularly minipreps that become unusable overnight at 4 C.

There is an ORF in the first reading frame of this sequence.

pSMPC186

SMPC186ECO

TTAGATCTTT TTTAAGCCAG CATACATCTA CCCCGGTTAA GATGTTGCAG
AATCTAGGAA GGGAATGGCG GCTTGAGAAC TTCCGAGTAA CATTCCCCTA
GTATGTATAA TCAACCGTCG AT

In general all efforts to subclone the BstY1 fragments from pSMPC172,181 and 185 particularly those suspected to contain the alpha structural region has been fruitless. We have attempted to subclone into pBR322, pIBI25, pZF18u and M13mp18. In all cases the double stranded DNA appears to be unstable and refuses to produce readable restriction fragments. In the case of the M13 subclones we were able to isolate single stranded DNA that gave appropriate mobilities when run on agarose gels but as with the other

vectors the double stranded form was unstable and produced no readable digestion patterns. In addition the single stranded form refused to sequence with either Klenow, Sequenase or Taq protocols regardless of the template or annealing procedure used.

There is no ORF for this sequence.

DISCUSSION

The genetic engineering of *Spirulina* has been found to present some rather formidable obstacles due to the nature of the organism. From the most basic techniques of plating for clonal colonies to DNA manipulation and transfer. The filamentous character makes it difficult to produce truly clonal colonies in that fragmentation appears to have a practical limit dictated by the rigid cell and cross walls that separate each cell. It would appear that the theoretical limit would be a 3-4 cell fragment such that the integrity of the 1 or 2 middle cells is maintained (Van Ekylenberg). In practice we have been able to form an average 6-10 cell filament that could be revived. Secondly, the filaments are motile and difficult to plate and maintain as single colonies without modification of the growth media, i.e. the removal of calcium(Abeliovich). It may be possible to use a motility inhabiter,rather than calcium starvation, to maintain discreet colonies during manipulation.

Classical microbial genetics for the isolation of auxotrophic markers has proven impractical (Tiboni, Takiguchi) if not impossible due to the obligate autotrophic nature of *Spirulina*. This characteristic has been studied in few organisms (Quayle, Whittenbury, Smith). It appears that the basis of this effect has to do with an

incomplete TCA cycle at the point of the alpha-keto-glutarate dehydrogenase, and NADH oxidase being either deficient or absent. The upshot being that excess (exogenous) amino acids overwhelm the capacity of the organism, causing a lethal accumulation of keto acids and ammonia, thus, mixotrophic lysis. There is however no published evidence of this metabolic break in *Spirulina*. The inability of Spirulina to grow in the presence of individual amino acids or sugars, and it's rapid lysis when placed on supplement media during conjugation trials, supports this theory.

This is not to say that mutants cannot be produced in Spirulina. Several amino acid analog resistant mutants have been evaluated (Tiboni) and the possibility of spontaneous morphologic mutants (Bai) exists. This suggests that a more imaginative approach need be taken to generate mutants with usable phenotypes.

Finally, in regard to general growth and strain characteristics, it is evident that a less than comprehensive study on growth effects has been done. It is well known that environmental and nutrient conditions have a potent effect on the general composition of Spirulina (VanEkylenberg) as well as other cyanobacteria. In some cases this is being elucidated on the transcriptional level (Tandeau De Marsac). Detailed composition analysis (Durant-Castil, Clements) has been done on various strains of

Spirulina with little or no regard to growth conditions. It may well be that many of the problems involved with cell manipulation on the molecular level, could be solved with the proper culture conditions. There is some evidence that nutrient starvation and/or environmental effects may be useful in effecting membrane composition (Campbell,Cohen, Reithman) for the purpose of transporting macromolecules such as in transformation. Our experiments, using cells growing on iron depleted media, showed an increased tolerance to chloramphenicol selection after a transformation procedure. We have not,however, been able to identify the presence of vector DNA by hybridization to genomic preps of tolerant filaments. This analysis was done on "mini preps" of test cultures and, as a rule, no consideration was given to low copy target DNA during any hybridization procedure.

The next level of problems has to do with the isolation and manipulation of plasmid and chromosomal DNA. The isolation problem, having to do with the rigidity of the cell wall and the exceptional amount of protein to be extracted, was resolved by Tiboni in 1985. The extensive proteinase treatment now seems an obvious solution to total DNA isolation. We have found that this procedure is only complete with density gradient centrifugation in order to remove pigments and polysaccharides that copurify with the DNA during extraction and precipitation. Following this

procedure we were able to obtain sufficient DNA that would digest reproducibly. It also may be that this rigorous treatment is responsible for the absence of any observable plasmid DNA. A DNAse contamination in the lytic enzymes or the release of cellular DNAse could mask the detection of low copy or mega plasmids. It seems unusual that no Spirulina species contains any plasmids. Whether this has to do with the lysis technique, the aforementioned culture conditions or the true character of the species is still unknown.

Nevertheless the DNA that is obtained is in itself quite unusual. It appears that the DNA of Spirulina is exceptionally resistant to restriction enzyme digestion and produce non-uniform patterns on inspection of stained gels. This is by no means an exhaustive study. It has been suggested that one round of density gradient centrufigation is not a guarantee of purity irregardless of OD 260/280. Our selection of enzymes were based only on those used in the literature and those most often found in multiple cloning sites. Most appear to have no effect on the DNA while a few appear to cut so frequently that no DNA is visible above 500bp. One is initially inclined to suggest a modified DNA such as extensive methylation. This factor cannot be totally ruled out although, our analysis does not indicate any unusual amount of modified nucleic acids. The %G/C is certainly not exceptional as is indicated from both

Tm and HPLC analysis. This data compares quite well with the literature (Ciferri, Herdman). The marine varieties, those grown in ES media are distinctly different from those that are grown in OT or brackish strains. One can only speculate as to the basis for the difficulty in digestion. Perhaps simply a coincidental selection of restriction enzymes.

Other anomalies having to do with Spirulina DNA, specifically the C-PC genes, is it's inability to hybridize with heterogolous DNA under any conditions that contain protein or polymer blocking agents. This will be discussed in depth later in this section.

The final requirement for genetic engineering is a method for mobilizing DNA(RNA) into the host such that it may be expressed. Here lies the core problem with Spirulina. At the onset of our investigation we approached this problem quite simply, in that we were attacking almost exclusively, transformation. Our reasoning for this was several fold. First the literature reported that *Spirulina* was not susceptible to any known cyanophage, eliminating transfection. Second, conjugal systems had not been observed in any cyanobacteria and, with what we had observed, did not seem feasible (i.e. incompatible growth conditions). Third, the system operating at that time for cyanobacteria was transformation which was compatible with the history of our lab. As far as a vector system, our plan

was to start with an established and/or original *E.coli*:cyanobacteria shuttle vector and work towards a modified system of our own. This involved generating a shot gun library of *Spirulina* in these vectors with the idea that an origin of replication or perhaps an elusive plasmid might be cloned. In addition we performed some DNA methylation experiments to protect the vector from potential restriction enzymes. To make the *Spirulina* competent, a barrage of conditions were explored ranging from simple physiologic conditions through calcium shock to elaborate spheroplast preparations and finally, iron starved cells. The obvious problems of restriction systems were not being ignored (Kawamura) so much as we hoped that under favorable conditions we might be able to transform enough cells that, either a companion methylation system would allow a few transformants to survive, or recombination between vector:insert and chromosome might incorporate a functional marker.

We now know that our "vector" DNA probably never survived the initial addition to the media. We have found an intensely powerful DNAse activity from both *S.maxima* and *S.platensis*. Using a simple permeabilization procedure on 1ml of culture we obtained a sample whose activity on DNA can only be compared to that of purified RNase on RNA. That is the degradation appears nonspecific and is very rapid even at high dilution and reduced temperatures. This

character should not be considered unique in that we have shown that Pseudanabaena, also an Oscillatoriaceae, has similar properties.

Here is where the focus of future work on *Spirulina* should begin. It should be determined wether this DNAse is truly nonspecific or a mixed bag of restriction enzymes. A rigorous program of mutagenisis and screening for a "DNAse minus" cell line would be tedious, but possible with the rapid screening procedure. With this in hand, transformation and/or conjugation trials could begin anew. This in addition to the previously mentioned culture effects (i.e. Fe starvation, photo adaption, etc.) may be helpful in producing a competent strain.

The problem of an appropriate vector may be resolved by the methods of Kolowsky and Lightfoot who managed to generate plasmid like vectors from insertion mutations derived from homologous recombinants containing an antibiotic marker.

Heterogolous Probes

As mentioned earlier, a good deal of difficulty was encountered in finding an appropriate hybridization condition for the phycobilisome system. It is therefor with some reservation that any broad conclusions be made based on one set of conditions. Nevertheless, work with the probes not associated with the phycocyanin gene have

presented some information. It should be mentioned that all of this work was done simultaneously with identical blots of UTEX2340 and 2342 DNA digests.

pSP301

This probe was a gift from Dr.Tiboni and contained the RuBISCo gene from a wild type *S.platensis*. The homology of this gene obviously is close to, if not 100%, and this was reflected in our results in that hybridization was positive regardless of the conditions used. Both UTEX2340 and 2342 gave apparent identical patterns. Due to the identical nature of the blots and, the previous publication of the cloning of the gene,we had little interest in this system and therefor did not pursue it further. Unfortunately, no publications have followed the initial report of this gene isolation. To the best of this authors knowledge, no DNA sequence data has been reported for Spirulina.

pRMR2

This probe is a clone of the nifHDK gene from Rhizobium and has been widely used in many systems, cyanobacterial and others, for the probing of the nitrogenase genes. It was of some interest to determine wether or not Spirulina carries a nonfunctional remnant of a nif system or simply is devoid of the gene. Since the probe is only a fragment of the nif gene (albeit an essential fragment) we can not say that there are no pieces of nif genes in Spirulina. The negative hybridization,

growth requirements and morphology and some relevant literature (Hamana) indicate that Spirulina may have never acquired or long ago lost the ability to fix nitrogen.

pTH228

This probe, a cosmid clone of the methionine synthase gene from Anacystis (Tandeau DeMarsac) was used more for a "let's do it and see what happens" reason than any thing else. We did manage to obtain some very weak signals on Spirulina digests that were virtually unreadable. If nothing else this verifies the fact that heterogolous probing is far from a routine technique and that homology of the target versus the probe DNA is not the only factor. It is difficult to imagine a reason for the lack of hybridization. One shudders to think that every time you shift from one gene to the next, a whole new set of hybridization conditions must be evaluated. In heterogolous hybridizations, there is no way of knowing the percent homology to insert into the Marmur equation and determine an appropriate "stringency" (salt concentration and temperature) for hybridization. One simply starts at a low "stringency" and systematically raises it to the highest level allowing hybridization. This research however strongly indicates that there is more to "stringency" than salt, temperature and formamide. Apparently "blocking" agents must also be considered as factors in hybridization.

pACYC184 and pUC-4K

These two probes are familiar cloning vectors and were used for their marker genes cam and kan respectively. Throughout this research we were exploring various techniques to transform Spirulina and were using pACYC184 or a derivative (pPUC29) as a vector. In a few cases we observed an increase in tolerance to cam with our treated cells, from 0 to 3ug/ml. We wanted to see if this increase could be traced to the presence of the vector in the cells. The results were negative and we therefor conclude that the cell line was an adaptive mutation or some type of contaminant protecting the cells.

Similarly no signals were obtained for the kan probe. Here we felt that an obscure plasmid or chromosomal marker might be uncovered. Our conclusions are that the resistance to kan is either a permeability factor or the antibiotic does not survive long enough in the alkaline media to have an effect.

In summary, our experiments indicate a few inconsistencies in standard hybridization parameters. Under the conditions used, we have shown positive signals for the RuBISCo, C-PC and methionine synthase genes. This involved using both homologous and heterogolous probes. No signals were obtained using the nif and kan probes indicating either no genes or no homology. The later explanation is a relative one. Without sequence data, relative homology is

indicated by the stringency of a hybridization and, stringency by ionic strength and temperature. There is then no obvious explanation why , two well conserved genes, RuBISCo and C-PC, should have such different hybridization requirements with little difference in the so called stringencies. The differences being primarily in the fact that one contains "blocking agents" and Tris, the other, phosphate and SDS. A similar situation has been reported recently ("BioTechniques",April 1989) involving improved signals on Northern blots. An explanation for these observations is not readily available but suggest a re-evaluation of classical hybridization theory is in order.

Sequencing

It is obvious by now that many obstacles exist between traditional techniques of molecular biology and functional methods for the *Spirulina* system. It should come as no surprise then that sequencing should be added to this list. However, the problems encountered appear, as with the hybridizations, to have more to do with the specific gene or region of interest than the character of *Spirulina* DNA as a whole (Riccardi,Tiboni).

We have successfully sequenced portions of the ends of our original C-PC clones. This data has not conclusively verified the identity of the clones. The fragments showing homology to the alpha-CPC probe (the 1.4 and 0.7kb BstY1

fragments) have proven resistant to all attempted methods of subcloning and sequencing. This is in part due to the apparent instability of the double stranded inserts. Potential recombinants, digested to release the insert have produced only a vector band and a background smear. Only a few of the single stranded M13 recombinants showed stable banding patterns on agarose gels that indicated inserts. This was true for two separate inserts (1.4 and 0.7kb) from two separate clones (pSMPC172 and 181). None of these alleged clones produced readable sequence data under any conditions. The background smear for each fragment size appears consistent. This is merely an observation on negative data but seems more than coincidental and was reproducible.

One can only speculate as to the cause for these problems. A possible reason may have to do with the location of the restriction sites in and around the C-PC gene. The fragments to be cloned may have strong secondary structures which are conserved under these digestion conditions. This structure if close enough to the sequencing primer region might prevent polymerization and stable enough to survive or revive from denaturing conditions. This type of strong secondary structure has been reported previously (Tandeau De Marsac 88) . It is proposed that hairpin loops immediately downstream form the alpha subunit are attenuated by a light regulated gene

product linked to the PC cluster. Together these structures are responsible for the photoregulation of the inducible transcripts.

The data that was obtained, although not definitive, is supportive of the validity of these isolates. The number of unique clones was at first alarming. In the progress of working up the isolates for sequencing, the literature produced more reports of multiple copy biliprotein genes. The cyanobacteria Calothrix has to date been identified with three C-PC genes and at least two PE genes (Tandeau De Marsac,1987). This happens to be the same bacterium that the clone pSMPC172Eco has shown homology to in the EMBL data search.

It is clear that a great deal more work need be done to answer the questions presented here in. It is the hope of this researcher that the opportunity and support, particularly from the commercial sector, that has been noticeable silent, presents itself such that at least some of these tasks can be accomplished.

Appendix A: Comparitive Nutritional Data on Protein Sources, per 100g

ITEM	RDA,70KG	SPIRULINA	BEEF	FISH	SOYA	YEAST	EGGS	DRIED MILK
CALORIES	3000		166	78	403	280	156	499
CARBOHYDRATES		15	0	0	33.5	38	0.8	38.2
PUFA			0.26	0.3	17.7	1	11	0.7
FIBER			0	0	4.9	2	0	0
PROTEIN	56	60	27	17.6	34.1	39	12.4	26.3
ILU (mg)		4100	1413	899	2012	2520	818	1592
LEU		5000	2213	1338	2898	3380	1092	2578
LYS		4000	2361	1549	2356	3450	794	2087
MET		2170	670	510	512	890	384	657
PHE		3950	1112	651	1841	2001	720	1271
THR		4170	1194	757	1466	248	620	1188
TRP		1130	315	176	512	740	200	371
VAL		6000	1501	933	1978	2870	918	1762
A (ug)	1000	400	0	0	80		1140	922
B6 (mg)	2.2	0.3						
B12 (ug)	3	200						
C (mg)	60	10.3	0	2			0	9
D (ug)	10		0				46	
E (mg)	10	19						
K (ug)	140	400						
BIOTIN (ug)	200	40						
folacin (ug)	400	50						
niacin (mg)	18	11	2.8	2.2	0.22	38		0.7
pantothinic (r	1.1							
riboflavin (m	1.6	4	55.3	0.07	0.31	4.3	0.26	1.21
thiamin (mg)	1.4	5.5	110	0.06	1.1	15.2	0.08	0.29
Ca (mg)	800	131	15.6	10	226	210	52	912
Cl (g)	5.1	0.44					52	
Cr (ug)	200							
Cu (mg)	3							
F (mg)	4							
Fe (mg)	10	58	4	0.4	8.4	17	2.2	1
K (g)	5.6	1.54	0.45	0.38	1.67	1.89	0.12	1.33
Mg (mg)	350	191	23.4	28	256	230	10	85
Mn (mg)	50	2.5						
Mo (mg)	15							
Na (g)	3.3	41	44	70	5	120	120	371
P (mg)	800	894	231	194	554	1750	200	776
Zn (mg)	15	3.9						

APPENDIX B

Hybridization Protocols Attempted

The following procedures were taken from relevant literature and referenced as such. Those not referenced are simply stringency modifications of previous methods or additions suggested during discussions with other researchers.

NITROCELLULOSE MEDIA

I ref Maniatis et.al. pg 326

1. wet baked filters in 6XSSC

2.transfer to prewash solution, incubate 42 C,1-2 hrs

prewash = Tris pH=8.0 50 mM

NaCl 1 mM

EDTA 1 mM

SDS 0.1 %

3.transfer filters to a sealed bag containing prehybridization buffer,incubate 42 C, 4-6hrs

prehyb buffer= formamide 50%

Denhardt's soln 5X

SSPE 5X

SDS 0.1%

salmon sperm DNA 100mg/ml

Denhardt's solution (50X)

Ficoll	5g
polyvinylpyrrolidone	5g
BSA	5g
H2O	to 500ml

SSPE (20X)

NaCl	175.3 g
NaH2PO4	27.6 g
EDTA	7.4 g
H2O	to 800ml
pH to 7.4 with NaOH	
H2O to 1l	

4. add to bag denatured 32P labeled DNA probe. Denature by heating to 100 C for %min

5.incubate 42 C for 1-3XCoT1/2 (usually over night=12hrs)

6.remove filters and wash 3-4X in 300-500 ml of 2XSSC with 0.1% SDS at room temp

7.wash 2X in 500ml 1XSSC,0.1% SDS 68 C, 1-1.5hrs each

8.if background still high, wash in 0.2XSSC,0.1%SDS 68 C, until removed

9.blot filters dry and expose to X-ray film at -70 C.

II ref Tiboni et.al.,J.Bact.159,407-9 (84)

1. prehybridize nitrocellulose blot for 5hrs at 42 C in the following solution 5ml per 15x15cm filter;

SSC	4X
formamide	50%
SDS	0.1%
Denhardts	5X
salmon sperm DNA	40ug/ml

2.the solution is replaced with same and denatured labeled probe, hybridized overnight at 42 C.

3. wash filter as described in method I

III ref G.Delequi, personal communication

1.soak nitrocellulose filters in 5X SSC and hybridize in

SSC	5X
formamide	43%
SDS	0.1%
Denhardt's	5X
sperm DNA	200ug/ml

37 C 24hrs

2.wash 2X in 5X SSC, 0.1% SDS room temp. 30min

3.wash 1X in 5X SSC, 0.1% SDS, 58 C

4. blot dry and expose

IV ref Pilot PNAS 81

1.prehybridize filter in

SSC	6X
Denhardts	10X
sperm DNA	200mg/ml

55 C 4hrs

2.add labeled probe (oligonucleotide) and hybridize at 37 C overnight.

3.wash 3X in 2X SSC room temp

4. air dry and expose

V

1.prehybridize by soaking in 2X SSC and then in hybridization buffer at 68 C for 3hrs

SSC	6X
Denhardt's	5X
SDS	0.5%
sperm DNA	500ug/ml

2. add 0.5 ug probe DNA (biotinylated pTP1 with cold pBR322 blocking agent) and hybridize 68 C overnight.

3. wash as in VI

VI

1. rehydrate nitrocellulose in 2X SSC

2.prehybridize at 42 C for 6hrs in

SSC	5X
formamide	50 %
Denhardt's	5X
Na2PO4	25mM
sperm DNA	500ug/ml

3.add probe and hybridize 42 C over night

4.wash as in IX

VII

1.prehybridize as in X

2.hybridize at 42 C 12hrs and room temp for 5hrs in

SSC	5X
Denhardts	1X
Na2PO4	20mM
PEG-4000	10%
sperm DNA	500ug/ml

and denatured labeled probe

3.wash as in X

VIII

1.prehybridize and hybridize as in XII

2.wash at room temp for 1hr each in the following

5X SSC

2X SSC (2X)

3.expose

IX

1.prehybridize nitrocellulose blot at 37 C 12hrs in

SSPE	5X
Denhardt's	5X
SDS	0.1%
sperm DNA	100ug/ml

2.hybridize at 37 C for 24hrs in

SSPE	5X
Denhardt's	5X
SDS	0.1%
sperm DNA	100ug/ml
PEG-4000	10%
labeled probe	

3.wash in 5X SSC 0.1% SDS 1hr room temp (3X)

4.expose

NYLON MEDIA

I ref BioRad bulletin 1234; hybridizations with DNA bound to 'Zetaprobe', K.C.Reed

1.prehybridize filters in the following solution for 0.5-24hrs in a sealed bag at 68 C. (approx. 1ml/cm2)

7.75 ml H2O

0.75 ml 20X SSPE

0.5 ml 20% SDS

0.5 ml 10% BLOTTO (=nonfat powered milk)

0.5 ml salmon sperm DNA 10mg/ml

2.for high sensitivity screening, replace the the solution with an equal volume of hybridization solution;

5.7 ml H20

2.0 ml 50% dextran sulfate (or PEG-6000)

0.75 ml 20X SSPE

0.5 ml 20% SDS

0.5 ml 10% BLOTTO

labeled DNA probe, denatured

3.hybridize at 68 C for 4-24hrs

4. remove the filters and rinse in 2X SSC and then wash them successively for 15min each at room temperature in the following; 2X SSC/0.1% SDS

0.5X SSC/0.1% SDS

0.1X SSC/0.1% SDS

5. finally wash in 0.1X SSC/1.0% SDS at 50 C for 30min.

6.rinse in 0.1X SSC, blot dry and expose to X-ray film.

II

1.prehybridize Zetaprobe electroblot after soaking in 6X SSC in the following

SSC	6X
SDS	0.5%
Denhardt's	5X

sperm DNA 100ug/ml

42 C, 3hrs

2.remove solution and hybridize in the following at 42 C 12hrs;

formamide 50%

Denhardt's 5X

SSC 4X

SDS 0.1%

sperm DNA 200ug/ml

3.remove filter and wash in 0.5X SSC, 0.1% SDS, %* C, 30min then 0.5X SSC 58 C , 15min

4.blot dry and expose

III NEN Colony/Plaque Screen

1.rehydrate nylon filters in 2X SSC

2.prehybridize at 65 C for 6hrs in

Dextran sulfate 10%

SDS 1%

NaCl 1M

3.add sperm DNA to 100ug/ml and labeled probe

4.hybridize 65 C 24hrs

5.wash 2X SSC (2X) 15min room temp

2X SSC 1% SDS (1X) 65 C 15min

0.1X SSC (1X) 15min room temp

IV

1.prehybridize nylon filter at 65 C 6hrs in

SSPE	1.5X
SDS	1%
BLOTTO	0.5%
sperm DNA	500ug/ml

2. add labeled probe (oligo) and hybridize overnight at 65 C

3.wash 2X SSC 0.1% SDS 15min room temp

0.5X SSC " " "

0.1X SSC " " "

4. expose to X-ray film

V

1.prehybridize at 65 C 4hrs in

SSPE	1.5X
PEG-4000	10%
SDS	1%
BLOTTO	0.5%
sperm DNA	500ug/ml

2.add labeled probe (oligo) and hybridize 65 C 24hrs

3.wash 15min ea at room temp in the following

2X SSC 0.1% SDS

0.5X SSC "

0.1X SSC "

0.1X SSC

VI

1.prehybridize nylon filters 60 C 24hrs in

SSPE	1.5X
SDS	1%
BLOTTO	0.5%
sperm DNA	500ug/ml

2.hybridize at 37 C for 24hrs in same as prehyb plus 10% PEG-6000 and labeled probe

3.wash for 30 min ea at room temp in the following

5X SSC 0.1% SDS

3X " " (2X)

3X SSC

VII

1.prehybridization at 65 C 12hrs

Zetaprobe		nitrocellulose	
SSPE	2X	SSC	6X
SDS	1%	SDS	0.5%
BLOTTO	0.5%	Denhardt's	5X
sperm DNA	500ug/ml	sperm DNA	500ug/ml

2.hybridization 37 C 12hrs

same with 10% PEG-6000	same with 10mM EDTA

3. wash 5X SSC 0.1% SDS 37 C 1hr

3X SSC 0.1% SDS room temp 1hr

3X SSC 0.1% SDS 37 C 1hr

2X SSC 0.1% SDS 42 C 1hr

APPENDIX C

SPIRULINA REVIEW

A. GROWTH, COMPOSITION AND MORPHOLOGY

The cyanobacteria *Spirulina* sp. falls into the section III group under the order Nostocales, family Oscillatoriaceae. The genus has also been previously classified under the name of *Arthrospira* sp. differentiating between species that have crosswalls and those that don't. However with improved microscopy it appears that all strains studied have crosswalls and the later term has fallen into disuse (Rippka).

Farrar has published a historical and anthropological study on the significance of *Spirulina* in the Lake Texcoco area of Mexico referred to as "tecuitlatl", the stones excrement. Dr. Farrar lists documents and letters of the Spanish conquest of the Aztec city Tenochtitlan on Lake Texcoco in Mexico.Letters from Diaz, Cortez and several friars document the consumption of a slime collected from the lake with nets, dried and sold in the market. The slime was described as a clear blue mud that breeds on the water. Although impossible to verify the slime was probably Spirulina.

There are at least two groups of *Spirulina* that have been identified as such, the marine strains and those that occur in brackish, highly alkaline lakes (in addition there appear to be some soil living strains but these are not well studied). There are few similarities between the two other than they are all cyanobacteria that form spiral nonbranching filaments. Marine strains are typically the smaller of the genera having a diameter of no more than 1 micron and short filament length of less than 100 microns. The brackish strains are much larger having a diameter of 3-10 microns and a variable filament length from the 10's of microns to several millimeters. Individual filaments are clearly visible to the naked eye. These are the strains that are of commercial interest and the ones that will be most often referred to in this thesis. The species classifications usually seen are;

Spirulina subsalsa, a marine strain

Spirulina platensis, both marine and brackish strains

Spirulina maxima, a brackish strain (also *S.gerileti*)

In the case of *Spirulina platensis*, the most predominant name in the literature, some confusion exists as to it's identity when wild isolates are used, which occurs more often than not. In order to avoid at least this one problem, we have acquired and have been consistently using isolates from the University of Texas at Austin culture collection. Two strains in particular have been the focus

of attention, they are *Spirulina platensis*, ID# UTEX 2340 and *Spirulina maxima*, ID# UTEX 2342. Both of these are brackish strains and will be referred to by number henceforth.

Strains 2340 and 2342 are very similar in their morphology and are only distinguished by slight differences in their spiral. In general they are of the larger, brackish variety, having a consistent diameter of about 5 microns and a variable length depending on the degree of agitation it experiences. Aerated liquid cultures usually fragment to an average filament length of a few millimeters or less. On agar plates however, the filaments can grow to several centimeters. These alga have distinct crosswalls that apparently are involved in the formation and degree of spiraling characteristic of the genus. Strain 2342 has a symmetrical spiral that has a large helix in the center and tapers toward the ends with a more or less regular pitch. This effect is only visible in the small to medium length filaments and is diminished in the longer ones. Strain 2340 has filaments of a regular helix with no observable taper but with a variable pitch that seems to be environmentally sensitive. The pitch can vary from a tight regular one of about 30 microns to a loose one that appears essentially straight. Both forms are observable together but one usually predominates over the other. It has been suggested by some that the linear form represents a spontaneous

mutant (Hall et.al.). This seems unlikely due to the fact that in our observations that changing the culture conditions from old to new media or from standing to aerated could reverse the process(Lewin).

Ecophysiologic adaptation is a very active process in these alga and has been thoroughly studied (Van Eykelenburg) in regards to appearance and disappearance of cellular structures and organelles in response to temperature, light and available nitrogen. This will be discused in more detail latter on.

The alga does not differentiate into heterocysts and will not fix nitrogen anaerobically as other species in it's family have been observed to do (Stewart). The alga does however appear to have a hydrogenase unrelated to a nitrogenase (Llama et.al. Hamana et.al., Gu). Other growth peculiarities are, that the alga thrives at relatively high pH (pH 9 at low density and >pH 11 for late log to stationary phase cultures). The organism also appears to be an obligate photoautotroph that is unable to form heterotrophic mutants (Riccardi et.al.). It was found that mixotrophic culture in no case enhanced chemotrophic conditions (Takiguchi et.al.) and was in most cases repressive at the millimolar level.

The central and south American countries have published a good deal on Spirulina production. One group of researchers describe a relatively inexpensive way to grow

Spirulina on a media composed primarily of seasalt and saltpeter(SSM). Carbonate, potassium phosphate and iron were supplemented. Production of dry biomass in the SSM was slightly less compared to a control media (Jimenez). The model study for optimized growth media for *S.platensis* was done by the Japanese (Ogawa). This is a follow up study on the media study done by Zuroke much earlier with some additions of micronutrients and an evaluation on heterotrophic and mixotrophic culture, the former being inhibitory and the later somewhat stimulatory in the case of the addition of 0.1% glucose and peptone.

One of the efforts in cultivation is to construct a bioreactor that would enhance the productivity of the culture. These constructs usually involve a contained flow system that primarily prevents the culture from becoming light limiting and allows for the efficient feed of gaseous carbon dioxide. Traditional batch harvest open pond systems with a depth of 20cm ,readily become light limiting and are able to grow up to 1g/l biomass but usually produce around 0.5g/l on a continuous basis. The contained systems do not become light limiting and are therefor able to produce up to 5g/l in batch culture and a optimal continuous flow harvesting of 1g/l (Leduy).

Santillian gives a general over view of traditional *Spirulina* production and a detailed listing of the nutritional composition of the Mexican variety *Spirulina*

maxima (geitleri). Some of the data is included in Appendix A.

The effects of mixotrophic growth have been best evaluated by Takiguchi. Phenylalanine and glutamic acid were most repressive on growth and could be reversed by the addition of a-ketoglutarate. All amino acids were repressive except for proline. "In the mixotrophic culture of *Spirulina platensis* the effect of amino acids on growth are summarized as follows:

1.*S.platensis* utilized ala, arg, asp, asn, cys, glu, ile, leu, met, orn, pro, ser, trp, and tyr. Most rapid growth was found with asp. gln,gly,his, lys, phe, thr, and val were not used

2.The growth on asp was inhibited remarkably by the addition of phe, glu or gln.

3.Growth inhibition by the addition of phe in asp medium was observed at mM concentration and was recovered by the addition of oxaloacetate, pyruvate, or valine. Experiments suggest that phe blocks the metabolic step from asp to oxaloacetate, but the activity of glutamine oxaloacetate transaminase, which was extracted from *S.platensis* as the cell free preparation, was not inhibited by phe.

4.Phe inhibited the utilization of arg, cys, glu, ile, leu, met, orn, pro, and ser.

5.The growth inhibition by glu in asp medium was recovered by the addition of oxaloacetic acid or a-ketoglutaric acid.

6.It was presumed that the growth inhibition by glu medium depended on the concentration of ammonium which was released from glu."

Contrary to this, in the case of *Spirulina* sp. there are several references in the literature reporting the commercial production of the alga on livestock waste as a low cost substitute for carbonate (Chung). Saxena reports on the cultivation of Spirulina on municipal sewage supplemented with bicarbonate and either nitrate or urea. The crude biochemical composition of the cultures were compared to that of synthetic media (Zarrouk). Little or no change was observed for nucleic acid and lipid content. The protein content went down (with a broad fluctuation in the aminoacid profile), carbohydrates went up and the overall yield was slightly lower. It is the experience of this author, and others that have been involved with production scale operations,that in no case has there been a successful cultivation of any *Spirulina* species on organic carbon. Those that have reported such, have either used a short term, small scale experiment or used the organic feed at such a high dilution that it contributed little to the growth and carbonate was added to maintain growth. In all cases contamination by other organisms is inevitable and ultimately lethal to the culture.

The condition of obligate autotrophy has been discussed by many authors but actually studied by very few

(Quayle, Smith, Whittenbury). In general it is agreed that autotrophy stems from a deficiency in one metabolic pathway or another. This may have to do with a transport barrier or an enzymatic limiting pathway. In the case of obligate autotrophy and the lack of amino acid auxotrophs, it has been suggested and in a few cases demonstrated, that there is an incomplete TCA cycle, specifically no detectable alpha-ketoglutarate dehydrogenase. More importantly in the case of cyanobacteria there appears to be a companion lack of $NADH_2$ oxidase. The low level of respiration and lack of ability to mediate the transfer of electrons from $NADH_2$ to oxygen makes heterotrophic growth virtually impossible. The presence of excess amino acids, that is anything over cellular production, places an insurmountable burden on the cell in the form of accumulated ketoacids and ammonia.

The majority of the publications on *Spirulina* involve mass culture techniques and the composition of various strains in regards to nutrition. Although the impetus for this research, these studies are in general not relevant to this research and will not be mentioned further.

I am obliged at this point to recognize the Italian research group of Ciferri and Tiboni. Probably the world authority on *Spirulina*, it's ecology, production and biochemistry, they have published two reviews on the topic. Their most important contributions have been in the genetics of the organism. Their induction of a few mutants

and cloning of several genes have initiated the application of molecular biology to this commercially important alga. The authors have compiled a collection of data from several sources. The genome size has been found to occur in multiples up to 6x of 1.2x10^9 daltons (2 Mbp) with a G/C content of 44 to 52 mol%. Pakhomova found that there was N6-dimethyl amino purine in Spirulina DNA. His assay of the nucleic acid content showed for *S.platensis* a G-C% of 48% and adenosine of 26%, and 0.36% for dimethyl adenosine. (The base pair differences are undoubtedly due to species differences.) The Italian group has explored various other aspects of the organism focusing primarily on production and protein synthesis including amino acid metabolism.Various amino acid analog and amino acid resistant mutants have been isolated and characterized. These however are relatively few when compared to those produced using other organisms that are more responsive to genetic manipulation.

The other most published aspect of *Spirulina* production is osmotic adjustment. *S.platensis* can, through conditioning adapt to salinities of 1.5X seawater (Warr).*Spirulina platensis* isolated from Lake Chad and grown in BG-11 media supplemented with bicarbonate and NaCl were found to adapt to concentrations of up to 150% of normal sea salt. The osmoticum response studied were glucosyl-glycerol and trehalose that were found to be

influenced both by salt concentration and temperature. In addition Tel-Or evaluated the adaption of the photosynthetic apparatus to salt. Several species of cyanobacteria including a marine Spirulina and *Spirulina platensis* CCC#1475/4a were evaluated for oxygen evolution/consumption and cytochrome c reduction as a percentage of control in response to NaCl concentration. It was found that the marine strain was least sensitive and *S.platensis* most sensitive to increased salt concentrations. However after culturing *S.platensis* in high salt media an increased tolerance was observed, significantly so for oxygen evolution and slightly for cytochrome-c reduction. Therefor it was demonstrated that salt tolerance could be acquired.

Ecophysiologic response has continuously held a great deal of attention in the study of cyanobacteria. This is also the case for *Spirulina* particularly in regard to the enhancement of the overall nutritional composition. The general composition (Clement) and more detailed profiles (Durand-Chastel) are documented. The later publication surveys the composition of *S.maxima* and *S.platensis* and reports values for ash,salts,metals, protein and aminoacids, fatty acids and nonsaponifiable lipids, sterols and caratenoids, carbohydrates, vitamins and heavy metals.

There are numerous documents on the composition of *Spirulina*, all of which give slightly different values.

Some of this has to do with strain differences. Since the primary commercial strains are the *S.platensis* or *S.maxima*, a good deal of this difference has to do with culture conditions. An early review by Al'bitskay covers the physiology, biochemistry and composition as a function of culture condition in *S.platensis*. The effects of light, temperature, nitrogen source and the difference between continuous and batch culture are investigated. When cultures were kept to a thickness of 0.8-1.0cm., optimum productivity was 0.15g/l-hr at a culture density of 3g/l, at 34 C under high (60klx) illumination. The product contained 62% protein. The biochemical composition (ie; crude fat, total carbohydrates, etc.) could be altered significantly by changes in the aforementioned parameters. Use of urea as a nitrogen source resulted in an increase in arginine and methionine.

In a similar study, a detailed microscopic evaluation of the inclusions in *S.platensis* was performed as a function of light, temperature and nitrogen levels as well as a study on the shape of the crosswalls as a determinant of helix pitch (Van Eykelenburg).

SCHEME 1. Relative abundance of organelles

TEMP	15-17			17-20			20-25			25-30			30-37		
[NO3]	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
CYANO-	1	2	3	1	1	1	1	1							
PHYCIN				L			L	L							
POLY-				3	2	2	1	1	1	1	1	1			1
GLUCAN										H	H	H			H
CYLINDRIC BODIES	1	1 L	1 L			1 L	1 L					1		1 H	1
CARBOXY-SOMES			1 H			1 H			1 H			1 H			1 H
MESOSOMES	1									1 H		1 H			

3= exuberant, 2= abundant, 1= present, no symbol= not detected;L= low light (<=2klux), H= high light (>=6klux) nitrate concentrations; L=3mM, M=30mM, H=120mM

"The ultra structure of *S.platensis* was studied in relation to temperature, light intensity, and nitrate concentration. The organism was able to grow in media supplied with nitrate in concentrations up to 250mM. High nitrate concentrations increased the yield and growth rate at temperatures above 35 C. Occurrence, distribution and abundance of cyanophycine granules, polyglucan granules, cylindrical bodies, carboxysomes and mesosomes varied widely in relation to the factors studied. At low temperatures (<17 C) cyanophycine was the abundant organelle, especially at high nitrate concentrations, whereas in the temp range of 17-20 C polyglucan was found in large quantities particularly at low nitrate concentrations. Special attention was paid to the cylindrical bodies, the ultrastructure of which was dependent on temp. Three types of ultrastructure were distinguished, each with several possible shapes".

Further studies by this author involve the isolation, electron microscopy and theoretical hypothesis on the shape of the crosswall peptidoglycan structure. It is presented as the determining factor of the helical shape of the filament and that it is adaptive in nature.

In addition there are several publications involving specific components that fluctuate in response to environmental conditions. Many of these components are carbon storage structures aside from glycogen. One such is poly-beta-hydroxybutyrate (Campbell).The authors found that *S.platensis* would accumulate beta-hydroxybutyrate to a level of 6% at stationary growth when exposed to high

levels of CO2. Previous studies in *Chlorogloca fritschii* showed a requirement for acetate for accumulation of the polymer while *S.platensis* shows no change with the addition of acetate.

The fatty acid content of 19 strains of Spirulina and their response to light and temperature was investigated (Cohen).All strains studied contained gamma-linolenic acid (GLA) in the range of 0.7 to 1.4% of the total biomass. Maximal GLA production in response to temperature was species dependent. For UTEX 2340 it was 30 C. Fatty acid content increased with temperature while the relative amount of polyunsaturated fatty acids decreased. High light intensities combined with high temperatures (38 C) did not effect composition but decreased the total fatty acids by as much as 46%.Fatty acids were isolated by hexane extraction of and aqueous suspension of methanol-acetyl chloride treated freeze-dried cells.

Evaluation of the carbohydrate composition of *S.platensis* reveals two unique structures (Shekharam). *Spirulina platensis* contains 13.6% carbohydrate, the sugar composition of which is comprised principally of glucose along with rhamnose, xylose, mannose, galactose and two unusual sugars. The latter were identified by a combination of GC-MS, NMR and de-O-methylation as, 2-O-methyl-L-rhamnose and 3-O-methyl-L-rhamnose. Water soluble polysaccharides were complex and heterogenous while acid

soluble polysaccharide was a homogeneous glucan. Although not investigated in this report, other investigators have shown that the carbohydrate profiles are influenced by growth conditions.

Few studies have been reported for Spirulina in the field however one involves nitrogen limitation in natural populations (Rijn). The authors evaluated several physiological indicators (chl a, phycocyanin, total protein and carbohydrates) as a function of nitrogen availability and vertical migration in response to available light (buoyancy). Carbon uptake was monitored by C14. The results involving nutrient and light limitation were observed with some interesting changes in polysaccharide content with nitrogen limitation and their turnover during dark periods. The authors conclude that polysaccharide turnover plays an important ecophysiological role in the cyanobacteria and may be involved in transformation in the cyanobacteria.

Two final aspects of adaption should be mentioned. One involves phycobiliprotein structures and their response to environmental factors, primarily light and nitrogen availability. The former has been discused in the introduction. The later has been addressed by Boussiba. The author suggests, and has been supported by others (Grossman) that the biliprotein C-phycocyanin levels change in accordance with available nitrogen, thus acting as a ready reserve of nitrogen when needed. The protein can make

up more than 15% of total cell protein and be reduced to 50% of it's original level without any noticeable change in the growth rate. A similar system is the carboxylase enzyme RuBISCo that can be stored as carboxysomes under conditions of nutritional abundance to be retrieved later.

One final aspect of adaption to be mentioned has to do with that of the morphology of Spirulina, specifically it's spiral. Previously it was mentioned that the helix of the filament is dictated by the peptidylglycan crosswall and that it is sensitive to environmental factors. Not all agree with this theory. In the past the helix was considered a factor in taxonomic differentiation. It has however been observed that within a so-called pure strain, nonhelical filaments appear. A debate exists as to wether these are adapted or mutated filaments or even contaminants. Bai presents a question of species composition or morphological transformation in *S.fusiformis* in which he concludes on the case for competition. He gives an extended thesis on the various species morphology and "morphones" of *Spirulina* focusing on the subspecies *S.fusiformis*. The author finds that irreversible spontaneous mutants form linear morphones from helical wild types, their occurrence and growth being culture dependent. Lewin reported spontaneous uncoiled variants in culture and subculture of *S.platensis*. He suggests that the irreversible uncoiling of the species argues against the

taxonomic separation of Spirulina from Oscillatoria simply on the basis of coiling. Wether these uncoiled "variants" are truly irreversible or mutants has yet to be proven.

B.MEMBRANES AND MOTILITY

It seems appropriate to group these two topics together in that the function and makeup of the membrane is directly responsible for motility. Although *Spirulina* has been classified as a "gliding" bacterium, very little has been done to determine the mechanism of this process. In fact the process of gliding is far from understood in any organism.

The spiral nature of the alga seems to assist in this process in that it is observed to drill through greater than 1% agar on high cell density plates. There are three schools of thought on the possible mechanism 1) it operates by microcilia (that are not observable with conventional micropreparation),2) a directional extrusion of mucilage propels the filament along a surface or 3) some other mode of a contractile nature drives the filament. There is some evidence in support of the third hypothesis

Abelivoich found that Ca2+ is required to trigger motility in *S.subsalsa*. Motility was measured as a tail-wagging motion in the filamentous marine species and it is both calcium and sodium dependent. Data indicates that a

two-site active transport system is present that is dicyclohexylcarbodiimide (DCCD) inhibited suggesting a membrane bound Ca-ATPase. Motility is pH dependent with an optimum at 8.5. However norepinephrine can reverse the inhibition of low pH from nil to 50% motility. Calcium shows a threshold for motility at 5mM however this can be reduced to submilimolar levels by aceylcholine. An analogy is drawn between these observations and those for smooth muscle.

In a unrelated study, support is found for the two-site transport mechanism which also can bind germanium, which is of clinical interest as an additive to the food source.Yanagimoto found that sulfate, Ca, and Ge uptake all involve a 2 site active transport system in *S.platensis.*

The sulfate uptake system shows an optimum transport at pH 9.0, a temperature of 40 C and a cell density of 2.5x10^6 cells/ml (Menon). Various photosynthetic and metabolic decouplers were found to drasticly reduce sulfate uptake thus demonstrating active transport. The process is inhibited by structural isomers such as molybdate and tungstate and is stimulated by sulfate starvation. Kinetic data show a two site transport system. One site being a low affinity, constitutive expressed permease and the other a high affinity inducible permease.

Finally, Riechenbach reports on the difference between gliding cyanobacteria and filamentous gliding heterotrophic

eubacteria by comparison of 16S rRNA fragment catalogs. A classical hypothesis exists, that certain gliding filamentous bacteria are apochlorotic cyanobacteria. This was tested in regard to matching of rRNA 6-17mer fragments. *Spirulina maxima* and *platensis* were the representitive cyanobacteria in comparison with a variety of eubacteria. No relation was evident and the assumption that motility can be used as a taxonomic indicator is apparently not valid. It may however be that *Spirulina* is unique in this respect. It should be remembered that Van Ekylenberg observed that the cylindrical bodies were often found attached to the crosswalls of the filament and may well be involved in a contractile process in concert with the crosswall resulting in a augar-like movement propelling the filament. This would be in contrast to the more popular theories on gliding such as microcillia or extrusion type propulsion.

C. ENZYMOLOGY

Most of the enzymology studies in Spirulina have to do with the photosynthetic apparatus. DeRoo studied the effect of cations on the induction of PSII in *S.platensis*.The effect is at least partially due to surface charge screening effects which permit freer access of anionic ferricyanide to the vesicle membrane surface.This is

further supported by the cation reversed effect of DBMIB on oxygen evolution and the trivalent specific reversal of DCMU on ferricyanide photoreduction. To study electron transfer, Robinson made spheroplast preparations of *S.platensis*.The impermeability of Spirulina to certain photosynthetic inhibitors (ferricyanide) was over come by the generation of spheroplasts prepared by a KCl/EDTA/lysozyme treatment that allowed for measurement of photosynthetic O2 evolution, photophosphorylation and electron transfer.

In a related study Kaplan investigated photoinhibition in *S.platensis*. The author proposes that the organism has the ability to store CO2 and protect itself from photoinhibition. Starving the cells of carbon or the addition of photoinhibitors causes a marked decrease in the oxygen produced. Addition of catalase reverses some of the inhibition. It is suggested that an accumulation of H_2O_2 is responsible for the inhibition.

Lerma has found an ATPase in *S.maxima* with unusual properties.This study on the isolation and purification of the thylakoid bound ATPase of *S.maxima* showed that the cyanobacterial enzyme has characteristics similar to those of the chloroplast. In addition, coupling factor AF1 was found to be unusual among mesophylic cyanobacteria in that it has a high Ca-dependent ATPase activity after heat treatment and is stable at room temperature.

Cytochromes (Gomez-Lojero) and ferredoxins have often been isolated from *Spirulina* due to their relative abundance. Ferredoxin and other halophylic proteins in *S.platensis* and various halophylic bacteria containing chloroplast-type ferredoxins,were subject to a theoretical evaluation of amino acid sequence data. from this is presented the idea that halophylic proteins substitute less bulky acid residues to counteract salt competition for water binding sites (Rao). This substitution does not apply to active site residues.

In regard to basic metabolism there are few publications on isolation and characterization of enzymes. The main interest in cyanobacteria has usually been in nitrogen fixation. *Spirulina* is not able to fix diatomic nitrogen either aerobic or anaerobically therefor is apparently devoid of functional *nif* genes. However a hydrogenase has been detected and purified from the algae. Although usually associated with the nitrogenase, several non-nif hydrogenases have been found in cyanobacteria as a component of PS-I. Independently first with *S.maxima* (Llama,1979) and then with *S.platensis* (Gu,1987), a non-nitrogenase (no ATPase) hydrogenase was isolated and purified. Gu et.al had demonstrated that *S.platensis* could evolve hydrogen gas and that it's production was inversely correlated to PSII oxygen evolution which poisons the hydrogenase. Further there appeared to be a competition for

reductant between the hydrogenase and nitrate reductase that could be eliminated by the use of urea as the nitrogen source.

Nitrogen metabolism has been evaluated both for inorganic and organic forms. The preferred nitrogen source for Spirulina is nitrate however urea is also readily assimilated. Carvajal reports on the purification of urease in *S.maxima* by DEAE and G-200 chromatography revealed a 38,000 MW protein that forms stable hexameric complexes (232,000 MW) that operate at a optimal pH of 8.7 with a Km of 0.12mM.

In regard to growth characteristics it was mentioned that certain amino acids were inhibitory to growth. Amino acid and protein turnover is the primary study for the Italian group of Riccardi, Tiboni et.al. They have recently published the detection and characterization of acetohydroxy acid synthase (AHAS) in *S.platensis*. In the report they show inhibition of *S.platensis* growth by valine and describe an AHAS activity from this organism. The experiments suggest that valine inhibition of *S.platensis* growth is exerted at two levels. Both lead to isoleucine starvation, block of protein synthesis, repression of biosynthesis and inhibition of AHAS activity. Neither leucine or isoleucine seem to be directly involved in the control of AHAS synthesis. The biochemical data suggest the existance of one AHAS activity in *S.platensis* with two

optimal pH values. This activity is inhibited by valine. Valine equally represses the production of enzyme activity at pH 6 and 7.5

Earlier studies by this group on the isolation and purification of the ribosomal subunit EF-Tu from *Spirulina platensis* allowed for it's characterization and comparison to that of *E.coli* (Tiboni). The protein is somewhat larger than that of E.coli at 49,000*d* but may be substituted for that of *E.coli* in in-vitro protein synthesizing systems and is sensitive to kirromycin, a antibiotic that specifically interacts with eubacterial EF-Tu.

Finally a publication of direct interest to this research identifies the restriction enzymes from *S.platensis ,siamese*. Three enzymes, two identical to Tth111I and Hae111 and one unique enzyme were identified (Kawamura). The unique enzyme was designated SplII and has the recognition sequence of C'GTACG. These three enzymes were isolated from total soluble protein and chromatographed on DEAE with NaCl gradients to purity.

D. GENETICS

Aside from one publication on the isolation of amino acid analog resistant NMG induced mutants, (an azetidine resistant mutant showing proline overproduction and increased salt tolerance (Kawamura)) all genetic and

molecular biology has come from the Italian lab (Riccardi, Tiboni et.al). Their work has touched on various aspects of Spirulina biochemistry including uptake, amino acid analog tolerance, osmoregulation and isolation of a mutated enzyme (methionyl-tRNA synthetase) and evaluation of it's altered activity. Further analysis of an ethionine resistant mutant showed an altered met-tRNA synthetase.

Later several significant metabolic genes have been identified and partially characterized by cloning from a cosmid library of *S.platensis* (DeRossi). Since 1984 Tiboni et.al. have published several articles on the detection , cloning and expression of those genes involved in protein synthesis including EF-Tu (tufA), EF-G (fus), ribosomal proteins S12 (str or rpsL) and S7 (rpsG) as well as the RUBISCo and glutamine synthetase genes. These genes have either been expressed in *E.coli* or their protein products were able to substitute for *E.coli* proteins in cell free systems. The library was also screened with heterogolous probes using the photosynthetic reaction center gene from *Anabaena* and the phycocyanin gene from *Agmenellum*, however in the case of the phycocyanin gene the clone was either unstable or a false positive and was never pursued further (Tiboni, personal communication).

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