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THE USE OF DNA-DNA HYBRIDIZATION FOR DETERMINATION OF THE RELATIONSHIP BETWEEN SOME BLUE-GREEN ALGAE (CYANOPHYCEAE)

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SUMMARY

A DNA-DNA hybridization membrane filter method suitable for blue-green algal DNA was developed. One of the main obstacles, the high amount of aspecific binding of the blue-green algal DNA to filters, could be resolved by means of an additional purification step involving isopycnic centrifugation of the thoroughly purified DNA. Application on DNA of several strains of blue-green algae indicates that this method is useful for taxonomic purposes.

1. INTRODUCTION

Blue-green algal taxonomy has been approached in different ways, all with one major goal: defining those phenotypic properties which are taxonomically useful. Because the phenotype is the product of genetic and environmental factors, the stability of certain characteristics is a function of the properties of the environment. This is sufficiently recognized: most recent authors reevaluate the usefulness of the characters for taxonomy, and revise the still currently used classical classification (GEITLER 1932). DROUET (1968, 1973) and DROUET & DAILY (1956) propose an extensively modified classification based on the assumption that most of the species described are ecophenes (ecological growth-forms), and that only few morphological characters can be used for classification. STANIER et al. (1971) have considerable doubts concerning the usefulness of morphological characters of the coccoid blue-green algae. They favour a set of other phenotypic, mostly biochemical, properties. In addition they also use the DNA base composition, a genotypic character previously used by EDELMAN et al. (1967), as a factor to obtain a more satisfactory classification.

We agree with Edelman et al., Stanier et al., and LEACH & HERDMAN (1973) that increasing knowledge concerning the genotypic relationship will lead to a more satisfactory classification of the blue-green algae. This knowledge can also be used to check Drouet's species concept, because this offers the opportunity to test whether organisms displaying different phenotypes are genotypically identical, or not (see discussion in STAM & HOLLEMAN 1975).

The determination of DNA base composition is of limited value in estab-

lishing taxonomical relationships. As has been shown by CRAIG et al. (1969) for *Anabaena variabilis* and *Nostoc muscorum*, DNA-DNA hybridization provides more reliable information.

The present paper concerns the development of a hybridization method suitable for blue-green algal DNA and application of this method to a number of blue-green algal strains belonging to Drouet's species 'Schizothrix calcicola'.

2. MATERIALS AND METHODS

2.1 Strains

Table 1 enumerates the blue-green algal strains used, to which in the following will be referred to by there strain numbers.

Table 1. Blue-green algal strains used (1).

strain number	species name attached to strain
426	*Phormidium luridum* var. *olivacea* Boresch
427	*Phormidium foveolarum* Gomont
482	*Plectonema notatum* Schmidle
485	*Plectonema* spec.
487	*Lyngbya* spec.
488	*Lyngbya* spec.
581	*Plectonema boryanum* Gomont
594	*Plectonema boryanum* Gomont
595	*Plectonema boryanum* Gomont
596	*Plectonema boryanum* Gomont
597	*Plectonema boryanum* Gomont
598	*Plectonema calothrichoides* Gomont
625	*Anacystis nidulans*
790	*Plectonema boryanum* Gomont

[1] All strains were obtained from the Indiana University Culture Collection (STARR 1964). The numbers used are Culture Collection numbers.

2.2 Growth conditions

Except for strain 625, all strains were grown in modefied Chu-10 medium as described before (STAM & HOLLEMAN 1975). If used for mass-culturing, to this medium 1 g/l tris(hydroxymethyl)aminomethane (TRIS) was added. This adjusted the pH to 8.5 before autoclaving. Cultures grown for the purpose of isolation of radioactive DNA contained only half the amount of K_2HPO_4. Strain 625 was grown in BG-11 medium described by STANIER et al. (1971).

Usually 50 ml of a three weeks old stock culture were added in a sterile way to a rubber-stoppered 5 litre culture flask containing 4.5 litre medium. Radioactive DNA was obtained from 750 ml cultures inoculated with 10 ml of stock culture.

The cultures were incubated for 3 to 4 weeks at about 23°C, 1500 lux (cool white fluorescent tube, Philips TL 20/34 de luxe) and continuously stirred electromagnetically. The cultures were aerated with 95% N_2 + 5% CO_2 during 8 hours of a 24 hours period. Cultures for the isolation of radioactive DNA contained five mC of ^{32}P, as Na_2HPO_4. The radioisotope was added three days before harvesting the cells.

2.3 DNA extraction

DNA was extracted essentially according to the method of GRAIG et al. (1969).

The cells were harvested by centrifugation and resuspended in a volume of 0.15 M NaCl + 0.015 M Na-citrate (SSC) equal to the packed volume of cells, and homogenized with a Teflon homogenizer to break up clusters of trichomes. Bacterial contamination was checked by microscopic observation. If present, bacterial cells were removed by repeated low speed centrifugation in sterile SSC until the number of bacterial cells was less than 3% of the total number of cells. Cells were counted with a Bürker counting chamber according to BURNHAM et al. (1973).

Filamentous strains were extruded through a French pressure cell (Sorvall) at 15.000–18.000 psi, or an X-press (Biotec, Sweden), followed by incubation for 15 min at 60°C with 2% sodium dodecyl sulfate (SDS) and 4% 4-aminosalicylate (PAS). Radioactive cells were desintegrated by sonification with an ultrasonic desintegrator (MSE 150 W, 2.5 min at 9 micron) and treated with 400 µg/ml lysozyme (BDH) for 30 min at 37°C before treatment with SDS and PAS. Coccoid strain 625 was lysed by successive treatment with 1 mg/ml lysozyme for 1 hr at 37°C, 2% SDS for 20 min at 60°C and with 0.5 mg/ml pronase (Calbiochem, grade B) overnight at 37°C, according to SHESTAKOV & KHYEN (1970). Pronase was preincubated during 3 hr at 37°C to remove possible traces of DN-ase.

Deproteinisation was started by shaking with an equal volume of phenol-m-cresol (140 ml redistilled m-cresol, 110 ml demineralized water, 1 g 8-hydroxyquinoline and 1 kg phenol p.a.) for 30 min. After centrifugation, the aqueous phase was pipetted off, from which the DNA was precipitated with an equal volume of 2-ethoxy-ethanol and dissolved in 0.1 × SSC (DSC). Deproteinisation was continued with another phenol-m-cresol treatment followed by shaking with an equal volume of chloroform-isoamylalcohol (24:1 v/v) for 15 min. The DNA was precipitated again and redissolved in DSC. RNA was digested by incubation with 50 µg/ml RN-ase (BDH) for 1 hr at 37°C. The RN-ase was preincubated during 10 min at 80°C to remove possible traces of DN-ase. Prior to a final deproteinisation step with phenol-m-cresol, the DNA solution was incubated overnight with 250 µg/ml pronase at 37°C. The DNA was precipitated once with 2-ethoxyethanol, twice with isopropyl alcohol (MARMUR 1961) and once more from 0.5 M NaCl containing Na-benzoate (30%) by the addition of an equal volume of 2-butoxy-ethanol. Precipitated DNA was always redissolved in DSC. Finally the DNA preparations were dialysed against SSC for 24 hr at 4°C. Dialyses bags were washed in 0.01 M

EDTA for 30 min at 100°C. The DNA in 1.5 M NaCl was stored at 4°C.

Bacillus subtilis (strain 168) DNA was extracted as described by VENEMA et al. (1965), with an additional phenol extraction after digestion with RN-ase.

Highly polymerized commercial calf-thymus DNA (BDH) was used.

2.4 DNA determination

DNA was determined spectrophotometrically by the method of GILES & MYERS (1965). Calf-thymus DNA was used as standard.

2.5 Isopycnic centrifugations

CsCl (suprapur, Merck) density gradient centrifugations were carried out in a Spinco model L2. For the estimation of the buoyant density of the DNA, DNA (20 μg) preparations in CsCl solution, adjusted to a refractive index at 20°C of 1.4008, were subjected to centrifugation in a SW 50 rotor at 30,000 rev/min for 68 hr at 20°C. The CsCl was dissolved in 0.05 M phosphate buffer (pH = 8) containing 0.01 M EDTA. Centrifugation tubes were pretreated with a 100 fold diluted Siliclad (Clay Adams) solution at 60°C, rinsed and dried.

The gradient was collected from the top by introducing at the bottom an almost saturated CsCl solution, passed through an U.V. cell (LKB Uvicord) and the absorbance was recorded (Honeywell Brown electronic, fig. 1). For the byoyant density determination, fractions were collected for refractive index measurements.

DNA base composition was calculated from the buoyant density according to SCHILDKRAUT et al. (1962) with the formula:

$$\rho = 1.660 + 0.098\,(GC)$$

and: $\rho = 10.860\ n_D - 13.500$

(ρ = buoyant density, n_D = refractive index)

To obtain high amounts of DNA for hybridization purposes, preparative centrifugations were carried out in a 40° fixed angle rotor with CsCl solutions containing 200–350 μg DNA. Fractions containing DNA were pooled and the solution was dialysed against two changes of SSC for 24 hr.

Molecular weights of extracted DNA's were determined by velocity sedimentation centrifugation in a Spinco model E analytical centrifuge equiped with an U.V. light source and photoelectric scanner as described by ARWERT & VENEMA (1973).

2.6 Hybridization procedures

In order to investigate which one is the more suitable for blue-green algal DNA's, we tested two generally known procedures for membrane filter hybridization of DNA: The PM-method (DERNHARDT 1966) and the DMSO-method described by LEGAULT-DEMARE et al. (1969).

Millipore HAWP 25 mm filters were used. Filtrations, including washing procedures, were carried out with the aid of a filter-block (New Brunswick). Each hybridization was done in triplicate or quadruplicate. DNA was heat denaturated in SSC for 10 min at 100°C followed by quick immersion in ice.

Hybridization temperatures were calculated according to DE LEY & TIJTGAT (1970), i.e. for the PM-method:

hybr. temp. = $T_{OR} = 0.51 \times \%GC + 47.0$

(T_{OR} = optimal renaturation rate temperature)

and for the DMSO-method:

hybr. temp. = $0.51 \times \%GC + 28.0$

Before denaturation, radioactive and competetive DNA were sheared with a Virtis homogenizer for 100 s at position 5.5 of the relative scale of the instrument, which reduces the molecular weight of the DNA to $5 - 6 \times 10^6$ (Arwert, personal communication). Radioactive DNA for release experiments (see section 2.6.5) was not sheared.

The filters were dried for approximately 15 min with the aid of an infrared lamp. The radioactivity on the filters was counted in 10 ml toluene + 0.5% (w/v) 2,5-diphenyloxazol (PPO) + 0.005% (w/v) 2,2-p-phenylen-bis-(5-phenyloxazol) (POPOP) in a Nuclear Chicago Mark II liquid scintillation spectrometer.

2.6.1 Fixation of DNA on filters.

Denaturated DNA was made up to 6 × SSC (six times concentrated solution of SSC) and passed slowly through a membrane filter, previously presoaked in 6 × SSC and washed with 50 ml 6 × SSC. Filters were loaded with 20 µg denaturated DNA. After washing with 5 ml 6 × SSC the filters were dried overnight at room temperature, and in a vacuum oven for 2 hr at 80 °C (GILLESPIE & SPIEGELMAN 1965).

2.6.2 The PM-method

Loaded filters were preincubated in vials with 2 ml of 0.02% each of Ficoll (Pharmacia), polyvinylpyrrolidone (Sigma) and bovine albumine (Armour) in 3 × SSC (three times concentrated solution of SSC) for 6 hr at 65 °C. Sheared denaturated radioactive DNA was added. The vials were transferred into a shaking waterbath for 16–24 hr at the temperature calculated. Hybridization was stopped by transferring the vials to ice. After rinsing in SSC, both sides of the filters were washed with 40 ml SSC by sunction filtration, punched to 18 mm diameter and dried. Blank filters were treated identically.

2.6.3 The DMSO-method

Loaded filters were put into vials with 2 ml 2 × SSC − 30% DMSO (twice concentrated solution of SSC containing 30% (v/v) dimethylsulfoxide; for hybridizations: Fluka puriss. p.a., for washing purposes: Baker) and sheared denaturated radioactive DNA was added. The vials were transferred into a shaking waterbath for 16–24 hr at the temperature calculated. Hybridization was stopped by transferring the vials to ice. The filters were rinsed in 2 × SSC − 30% DMSO, washed on one side with 50 ml 2 × SSC − 30% DMSO and on the other side with 100 ml 2 × SSC by sunction filtration, punched to 18 mm diameter and dried. Blank filters were treated likewise.

2.6.4 Competition hybridization
Filter-fixed DNA from strain 594 was hybridized with 5 μg ^{32}P-DNA of the same strain in the presence of various amounts of sheared denaturated competitive DNA. Only the DMSO-method was used.

2.6.5 Release of filter-fixed DNA
Filters were loaded with 20 μg denaturated radioactive DNA. Both after the fixation procedure and after the complete hybridization procedure the residual radioactivity on the filters was counted.

2.7 Statistics
Differences in the mean values of bound radioactivity in the hybridization experiments were tested with the aid of the Student's t test. The difference was assumed to be real at 5% level.

Sample variances were tested to be similar at 5% level for $n_1 - 1$ (v_1) and $n_2 - 1$ (v_2) degrees of freedom by the variance ratio test (F-test):

$$F = \frac{\text{greater variance estimated}}{\text{lesser variance estimated}} = \frac{s_1^2}{s_2^2} \quad (s_1^2 \geqslant [s_2^2])$$

When the variances were dissimilar, the degrees of freedom (f) for the Student's test were calculated with the formula:

$$f = \frac{(s_1^2/n_1 + s_2^2/n_2)^2 (n_1 - 1)(n_2 - 1)}{(n_2 - 1)(s_1^2/n_1^2)^2 + (n_1 - 1)(s_2^2/n_2^2)^2}$$

All formulas are according to De Jonge (1960).

3. Results

3.1 Growth conditions
When using non TRIS-buffered (Nicols 1973) media, the 4.5 l cultures of the filamentous strains frequently did not start to grow and the inoculum died off. This was caused by a fall of the pH from 8–9 to less than 6 during the first few days of incubation.

3.2 Bacteral contamination
Because strains 427, 482, 595 and 596 are non-axenic, we attempted to remove the bacteria selectively from the contaminated strains. After trying treatment with penicillin or chloramphenicol, transferring small pieces of agar with one or a few trichomes into small volumes of fresh media or onto freshly poured agar plates, dilution to such an extent that contaminants are not present in the final dilution, centrifugation through sucrose or rhenografin gradients and irradiation with U.V., all of which scored relatively little succes (Vance 1966,

WIEDEMAN et al. 1964, BROWN 1962, CAHN & FOX 1968 and HADDEN & NESTER 1968), we finally adopted the method of repeated low speed centrifugation. Especially if combined with aeration of the cultures with 95% N_2 + 5% CO_2 (which prevents explosive growth of bacteria), this method results in the reduction of the contamination to acceptable levels.

Purification of the blue-green algae from bacterial contamination is crucial for obtaining reliable data on DNA-DNA hybridization as is shown by the following: DNA was isolated from an axenic culture of strain 485 and from a culture of 485 for which no precautions were taken to keep it axenic. This culture contained approximately 30% bacterial cells. The two DNA preparations were both subjected to isopycnic centrifugation and used as denaturated filter-fixed substrate for hybridization with ^{32}P-DNA obtained from strain 426. The results of the isopycnic centrifugation (*fig. 1*) indicate that the contaminating DNA is detectable as a shoulder on the absorption profile. The contaminating DNA also interfered with the hybridization. It was observed that the hybridization was 18% less efficient with the contaminated DNA than with non-contaminated DNA (results not shown).

Repeated low-speed centrifugations usually reduce the number of bacterial cells to less than 3% of the total cell number. The extent of bacterial cells was determined by means of microscopic observation, according to STANIER et al. (1971). Because the bacterial cell and the blue-green algal one contain almost the same amount of DNA (CRAIG et al. 1962, KUNG et al. 1972), the DNA preparations are contaminated with less than 3% bacterial DNA. When these DNA preparations are subjected to isopycnic centrifugation, the U.V. pattern is identical to *fig. 1a*, and, when hybridized with ^{32}P-DNA from strain 426,

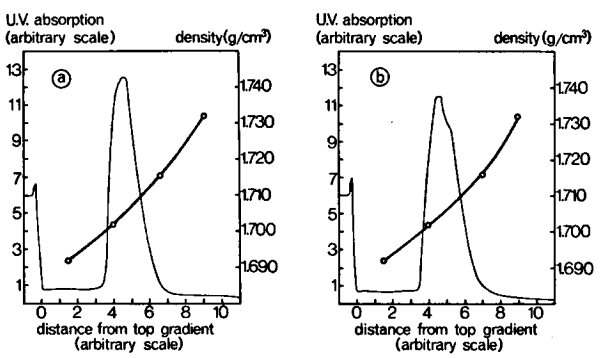

Fig. 1. Ultra-violet light absorption and buoyant density of DNA as a function of the distance from the top after centrifugation in CsCl. DNA from strain 485 (200 µg) was centrifugated in the 40 fixed angle rotor of a Beckmann preparative ultra-centrifuge for 64 hr at 30,000 rev/min at 20°C.

——— UV absorption tracing

o——o buoyant density of CsCl

a. DNA isolated from an axenic culture of strain 485.

b. DNA isolated from a culture of strain 485 contaminated with bacteria.

the level of hybridization of this DNA and the non-contaminated DNA do not significantly differ from each other (results not shown).

When the reduction of contaminating bacteria to less than 3% could not be achieved, the culture was not used for the isolation of DNA. For the isolation of radioactive DNA axenic cultures were used exclusively.

3.3 DNA purification

3.3.1 Optimal conditions for the isolation of DNA

The conditions for obtaining sufficient amounts of DNA differ for the filamentous and coccoid strains.

Although the filamentous strains do lyse when they are succesively treated mechanically (X-press or French-press), with lysozyme and detergents, little if any DNA is obtained. When the lysozyme step is omitted, DNA yields are good. If cell breakage is accomplished by ultrasonic desintegration, cells should additionally be treated with lysozyme in order to obtain lysates from which sufficient quantities of DNA can be obtained.

No DNA is obtained from the coccoid strain when mechanical treatment is followed by incubation with lysozyme and/or detergent. This confirms the observation of SHESTAKOV & KHYEN (1970), that cellbreakage by lysozyme and treatment with detergent yield satisfactory quantities of DNA.

3.3.2 Molecular weight of DNA

Purified DNA for fixation on filters had an S-value of 26.1, corresponding to a molecular weight of 1.5×10^7.

3.4 Fixation of DNA on filters

The amount DNA retained on the filter after the fixation procedure was 70–75% of the input. The effective amount of filter fixed DNA available for hybridization was therefore 14.5 μg per filter.

3.5 Selection of DNA-DNA hybridization method

Ideally, the effective amount of DNA fixed to the filter should not be subject to change during the hybridization experiment. However some loss is inevitable (DE LEY & TYTGAT 1970). In order to select the best method, we compared the release of filter fixed DNA at the hybridization temperature used in the PM-method and the DMSO-method. The results (*table 2*) show that the relatively high temperature used in the PM-method releases the majority of the fixed DNA from the filters, and the loss is greatly reduced at the temperature used in the DMSO-method. On the bases of these results and in agreement with the recommendations of DE LEY & TYTGAT (1970), we selected the DMSO-method for further experiments.

Table 2. The effect of temperature on the release of filter fixed DNA. Filters fixed with an average of 14.5 µg denaturated ^{3}H-DNA from *B. subtilis*, or isopycnicly purified denaturated ^{32}P-DNA from strain 594 were incubated for 16 hr at temperatures specific to the PM-method and the DMSO-method. The incubation temperatures were calculated as given in section 2.6.

DNA origin	hybridization method	incubation temperature (°C)	DNA release (%)
B. subtilis	PM-method	68	82.5
B. subtilis	DMSO-method	49	33.4
strain 594	PM-method	71.5	63.1
strain 594	DMSO-method	52.5	31.6

3.6 Hybridization blanks

3.6.1 Aspecific binding of DNA to filters

Because the amount of radioactive DNA on hybridization blanks (filters which do not carry single stranded DNA) should be as small as possible after having been exposed to conditions identical to the experimental filters, we investigated the effect of certain properties of the apparatus used. In addition, we studied the effect of purification of DNA by means of CsCl on the amount of association of denaturated radioactive DNA to the filter.

During hybridization the entire filter is exposed to denaturated radioactive DNA, but during the washing procedure, when the filter is tightened in the filter-block the circumferential margin is less accessible to the washing fluid than the remainder of the filter, which may result in less effective removal of aspecifically bound DNA from the margin. *Table 3* shows the effect of removal of 3.5 mm from the margin by punching. The amount of aspecific bound DNA can be reduced by a factor of approximately 9 when *B. subtilis* DNA is used, which, even in the absence of punching, gives acceptable background levels of aspecifically absorbed DNA.

Table 3 also shows that in the case of blue-green algal DNA, punching reduces the amount of aspecifically bound DNA by a factor of approximately

Table 3. The effect of purity of DNA and of filter treatment on the absorption of denaturated DNA on blank filters. Incubation and washing were according to the DMSO-method (see section 2.6.3). Five µg denaturated ^{32}P-DNA from strain 594 were incubated with blank filters for 16 hr at 49 °C and 52.5 °C respectively.

DNA origin	CsCl purification	punching of filters	association of input DNA (%)
B. subtilis	no	no	0.55
B. subtilis	no	yes	0.06
strain 594	no	no	5.54
strain 594	no	yes	1.24
strain 594	yes	yes	0.08

4.5, but even then the background level is still inacceptably high, presumably indicating that some contaminating factor is still present in the DNA which facilitates aspecific binding. In order to investigate this possibility, we purified the DNA further by isopycnic gradient centrifugation and tested the degree of aspecific binding of this purified DNA. *Table 3* shows that the isopycnic centrifugation reduces the amount of aspecific DNA binding to an acceptable level.

3.7 Competition hybridizations

Competition hybridization offers one great advantage: DNA from only one single preparation has to be fixed to the filter, while the direct DNA-DNA hybridization as described in the foregoing sections, has to be carried out with filter-fixed DNA from different preparations, which is likely to increase the variability of the experimental data.

In order to investigate whether this method is also applicable to the present DNA systems we examined the effect of homologous and heterologous DNA on the amount of hybridization. *Fig. 2* shows that the non-homologous calf-thymus DNA has no effect on the hybridization of the radioactive DNA from strain 594, whereas this hybridization decreases almost linearely with increasing amounts of non-radioactive DNA from strain 594 (and also with DNA from strain 482). This shows the suitability of the competetive version of the DMSO-method.

For further experiments we limited ourselves to testing competition with 100 µg, and calculated the DNA relatedness with the formula:

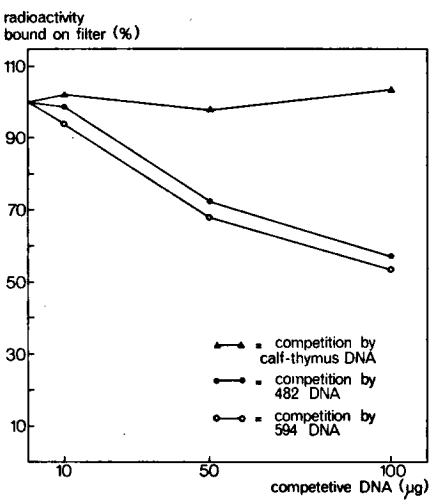

Fig. 2. The effect of homologous and heterologous DNA on hybridization of radioactive DNA. Filters fixed with an average of 14.5 µg denaturated DNA from strain 594 were incubated for 16 hours at 52.5°C with 5 µg denaturated ^{32}P-DNA from strain 594, and varying amounts of denaturated non-radioactive DNA from strain 594 and 482, and from calf-thymus. The hybridization and washing procedures were according to the DMSO-method.

$$\text{DNA relatedness} = \frac{C^{th} - C^{het}}{C^{th} - C^{hom}} \cdot 100, \text{ in which}$$

C^{th} = bound radioactivity by competition with 100 µg calf-thymus DNA.
C^{het} = bound radioactivity by competition with 100µg heterologous DNA.
C^{hom} = bound radioactivity by competition with 100 µg homologous DNA.

3.8 Use of the method for taxonomy

3.8.1 Direct DNA-DNA hybridization

When the DNA's from the blue-green algal strains listed in *table 1* are fixed on filters and hybridized with radioactive DNA from strain 594 and 790, they all, except strain 625, bind amounts of radioactive DNA from strain 594 and 790 which do not differ significantly from the amounts of DNA bound in the homologous hybridizations, as shown in *table 4*. This implies that no genotypical differences between these strains are detectable with this method. DNA from

Table 4. Hybridization of 5µg denaturated ^{32}P-DNA from strain 594 and 790 with an average of 14.5 µg denaturated filter fixed DNA from the blue-green algal strains listed in *table 1*, *Bacillus subtilis* and calf-thymus. Incubation and washing according to the DMSO-method. The data are expressed as % DNA relatedness versus 594 and 790, respectively. The data were subjected to a statistical analysis according to the formulas given in section 2.6.1. DNA base compositions (expressed as % GC) are listed.

strain number	% relatedness versus 594	sign. diff.	% relatedness versus 790	sign. diff.	% GC
426	99.5	–	97.6	–	47[1]
427	95.9	–	98.8	–	
482	102.9	–	103.6	–	
485	112.1	–	109.0	–	
487	98.4	–	110.3	–	47[1]
488	99.9	–	105.5	–	
581	98.0	–	100.9	–	48[1]
594	100.0		101.9	–	47–48[3]
595	101.3	–	96.8	–	
596	95.3	–	100.6	–	
597	106.7	–	101.6	–	
598	98.8	–	96.0	–	48[1]
790	108.3	–	100.0		47–48[3]
625	6.3	+	6.0	+	57[2]
B. subtilis	5.6	+	5.1	+	44[4]
calf-thymus	1.7	+	n.t.		

– = mean value *not* significantly differing from the mean value of the homologous (strain 594 or 790) hybridization.
+ = mean value significantly differing from the mean value of the homologous hybridization.
n.t. = not tested.
[1] EDELMAN et al. (1967).
[2] KUNG et al. (1972).
[3] Own determination.
[4] ARWERT & VENEMA (1973).

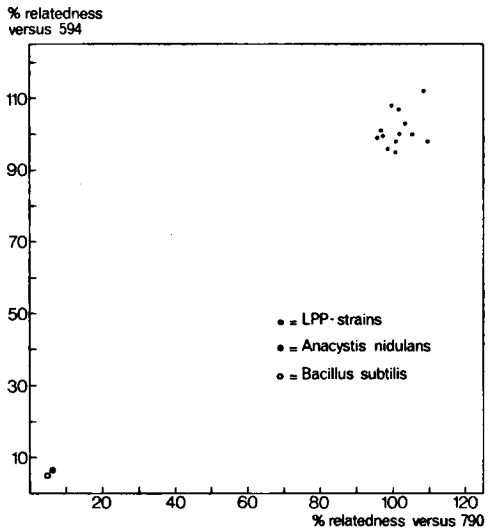

Fig. 3. Relation between relative relatednesses of the DNA's from the blue-green algal strains listed in *table 1* and from *Bacillus subtilis* versus DNA from strain 594, and of these DNA's versus DNA from strain 790. The relatedness was obtained from *table 4*.

strain 625 binds only about 6% of the 594 and 790 DNA, almost as little as DNA from *B. subtilis*. *Table 4* also shows that calf-thymus DNA hardly binds any 594 DNA.

Fig. 3 presents the data from *table 4* in a different way. It is clear that the *Lyngbya*, *Plectonema* and *Phormidium* strains (LPP-strains) cluster together and that *A. nidulans* and *B. subtilis* are clearly separated from the cluster.

3.8.2 Competitive hybridization

Table 5 shows the results of competitive hybridization of DNA from strain 594 in the presence of the DNA's from the LPP-strains, *A. nidulans*, *B. subtilis* and calf-thymus. The results indicate the same type of relationship as determined from the non-competitive hybridization experiments presented in *table 4*, indicating that the competitive hybridization procedure provides usefull data on the genotypic relationship of blue-green algae.

The DNA's from the LPP-strains compete not significantly different as DNA from strain 594, whereas *A.nidulans* DNA has much less influence on the homologous 594 hybridization. The influence of *Bacillus subtilis* DNA is almost negligible. The competitive effect of calf-thymus DNA was arbitrarely set at 0% (cf. section 3.7).

Table 5. Competitive hybridization. Filters fixed with an average of 14.5 µg denaturated DNA from strain 594 were incubated during 16 hours at 52.5°C in 2 × SSC–30% DMSO with 5 µg denaturated ^{32}P-DNA from the same strain, and 100 µg of competetive denaturated DNA from the strains listed in *table 1*. *B. subtilis* and calf-thymus. The relatedness is calculated according to the formula given in section 3.7. The data were subjected to a statistical analysis according to the formulas given in section 2.6.1.

strain number	% relatedness versus 594	sign. diff.
426	106.0	–
427	107.0	–
482	92.9	–
485	97.0	–
487	104.7	–
488	106.1	–
581	107.4	–
594	100.0	
595	96.3	–
596	101.5	–
597	96.3	–
598	100.0	–
790	100.3	–
625	6.2	+
B. subtilis	2.7	+
calf-thymus	0	+

– = mean value *not* significantly differing from the mean value of the homologous (strain 594) hybridization.
+ = mean value significantly differing from the mean value of the homologous hybridization.

4. DISCUSSION

Preferentially in DNA-DNA hybridization studies the DNA obtained from blue-green algal cultures should be free of contaminating DNA from other sources. In a number of cases this is difficult to achieve. Although evidence has accumulated during the last decade that the isolation of axenic blue-green algal cultures is possible (STANIER et al. 1971, CARR et al. 1973), we did not succeed in obtaining axenic cultures from strains 427, 482, 595 and 596, despite the application of several methods. This study shows that almost non-contaminated blue-green algal DNA can be obtained from those species which are resistant to those methods by repeated rounds of low speed centrifugations. Therefore DNA-DNA hybridizations involving blue-green algal DNA need not to be restricted to species from which axenic cultures are available.

Our results do not agree completely with those obtained by CRESPI et al. (1962) and EDELMAN et al. (1967) as far as complete lysis with the aid of lysozyme of the filamentous strains is concerned. In our hands mechanical treatment is indispensable in order to obtain complete lysis. This may well be due to varia-

tions in the conditions of growth of the cultures and in the age of the organisms harvested.

The extent of binding of the unlabelled DNA to the filters (see section 3.5) is comparable to that reported by DE LEY & TIJTGAT (1970), but is substantially less than reported by DERNHARDT (1966) and LEGAUT-DEMARE et al. (1967) for bacteriophage DNA. Possibly these differences are caused by difference in molecular weight of the bacteriophage DNA and the DNA used here.

DE LEY & TIJTGAT (1970) have shown that the most reliable hybridization temperature for determining the degree of DNA relatedness depends both on the hybridization method and the organisms investigated. These authors have shown that temperatures in excess of 70 °C render the results unreliable because of the release of high amounts of filter fixed DNA from the filter. Extensive release of filter fixed DNA at high temperatures is supported by our results (*table 2*). Because of these high losses, we decided to use the DMSO-method, which effectively reduces the loss; however, still a substantial amount of filter fixed DNA is released during incubation.

Because high amounts of aspecifically bound radioactive DNA will seriously interfere with a proper determination of the degree of DNA relatedness, it is essential that aspecific binding is reduced to negligible quantities. The results show that without further purification such requirements are easily fulfilled with bacterial (in the present case *B. subtilis*) or bacteriophage DNA. Surprisingly, the DNA isolated from blue-green algae by procedures aimed at attaining highly purified DNA produces high blanks. It is clear from the results presented in *table 3* that a final isopynic centrifugation step reduces the amount of aspecifically bound radioactivity to values comparable to the bacterial and bacteriophage systems. Apparently, either the isopycnic centrifugation step removes a factor that has both affinity to DNA and to the filter, or in DNA preparations from blue-green algae radioactive phosphate containing substance(s) are present, which are also capable of binding to nitrocellulose filters. Although we are not in the position to distinguish between these two alternatives, we favour the second explanation because polyphosphate is present in blue-green algae, especially after phosphate addition subsequent to a period of relative phosphate starvation (HAROLD 1966, Simon personal communication). This suggestion is supported by the observation that radioactive material is present at the bottom of the centrifuge tube after isopycnic centrifugation of the radioactive DNA, which does not absorb ultra-violet radiation. Possibly this material is polyphosphate of high molecular weight.

As shown in section 3.8 the hybridization method (including the competitive version) we developed can be used for the determination of the genotypic relationship between blue-green algae. For the strains investigated this leads to the following conclusions: The LPP-strains are genotypically identical, which is in agreement with their being morphologically identical (STAM & HOLLEMAN 1975), their susceptibility to the cyanophage LPP-1 (SAFFERMAN & MORRIS 1963, 1964) and the known DNA base compositions. Therefore the LPP-strains investigated have to be considered as representatives of one and the same

blue-green algal species.

This conclusion does not permit extrapolation to other strains classified in the genera *Lyngbya*, *Plectonema* and *Phormidium*. We assume that the strains investigated in this study were identified incorrectly (as far as the *Lyngbya* and *Plectonema* strains are concerned), and that these strains belong to the genus *Phormidium*.

According to DROUET (1963, p. 270, footnote 10) the *A. nidulans* strains 625 is an ecophene of *S. calcicola*. Drouet also assumes that strain 426, 427, 488, 581, 594, 597 and 598 are ecophenes of *S. calcicola*. This would imply that strain 625 should be genotypically identical to those strains. The present results clearly show that the assumption of Drouet with respect to the classification of *A. nidulans* is incorrect.

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REFERENCES

ARWERT, F. & G. VENEMA (1973): Transformation in Bacillus subtilis, fate of newly introduced transforming DNA. *Molec. gen. Genet.* **123**: 185–198.

BROWN, Jr, R. M. & H. W. BISCHOFF (1962): A new and useful method for obtaining axenic cultures of algae. *Phycol. News Bull.* **15** (4): 43–45.

BURNHAM, J. C., T. STETAK & J. BOULGER (1973): An improved method of cell enumeration for filamentous algae and bacteria. *J. Phycol.* **9**: 346–349.

CAHN, F. H. & M. S. FOX (1968): Fractionation of transformable bacteria from competent cultures of Bacillus subtilis on rhenografin gradients. *J. Bacteriol* **95** (3): 867–875.

CARR, N. G., J. KOMAREK & B. A. WHITTON (1973): Notes on isolation and laboratory culture. In: N. G. CARR & B. A. WHITTON (eds.), *Botanical Monographs* **9**: 525–531. Blackwell Scientific Publications, Oxford-London-Edinburgh-Melbourne.

CRAIG, I. W., C. K. LEACH & N. G. CARR (1962): Studies with deoxyribonucleic acid from blue-green algae. *Arch. Mikrobiol.* **65**: 218–227.

CRESPI, H. L., U. SMITH & J. J. KATZ (1972): The action of lysozyme on several blue-green algae. *Biochem. Biophys. Res. Commun.* **9**: 569–577.

DERNHARDT, D. T. (1966): A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* **23** (5): 641–646.

DROUET, F. (1963): Ecophenes of Schizothrix calcicola (Oscillatoriaceae). *Proc. Acad. Natur. Sci. Phildelphia.* **115** (9): 261–281.

— (1968): Revision of the classification of the Oscillatoriaceae. *Monogr. Acad. Natur. Sci. Philadelphia.* Fulton Press Inc., Lancaster, Pennsylvania.

— (1973): *Revision of the Nostocaceae with cylidrical trichomes.* Hafner Press, New York-London.

— & W. A. DAILY (1956): Revision of the coccoid Myxophyceae. *Butler Univ. Bot. Stud.* **10**.

EDELMAN, M., D. SWINTON, J. A. SCHIFF, H. T. EPSTEIN & B. ZELDIN (1967): Deoxyribonucleic acid of the blue-green algae (Cyanophyta). *Bacteriol. Rev.* **31**: 315–331.

GEITLER, L. (1932): Cyanophyceae. RABENHORST'S *Kryptogamen Flora* **14**, Akad. Verlaggeselschaft, Leipzig.

GILES, K. W. & A. MYERS (1965): An improved diphenylamine method for the estimation of deoxyribonucleic acid. *Nature* **4979**: 93.

GILLESPIE, D. & S. SPIEGELMAN (1965): A quantitative assay for DNA/RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* **12**: 829–842.

HADDEN, C. T. & E. W. NESTER (1968): Purification of competent cells in the Bacillus subtilis transformation system. *J. Bacteriol.* **95** (3): 876–885.

HAROLD, F. M. (1966): Inorganic polyphosphates in biology: Structure, metabolism and function. *Bacteriol. Rev.* **30** (4): 772–794.

JONGE, H. DE (1960): Inleiding tot de medische statistiek, deel 2. *Verhandelingen van het Ned. Instituut voor praeventieve geneeskunde* **48**.

KUNG, S. D., M. C. MOSCARELLO & J. P. WILLIAMS (1972): Studies with deoxyribonucleic acid from blue-green algae. *Plant Physiol.* **49**: 331–334.

LEACH, C. K. & M. HERDMAN (1973): Structure and function of nucleic acids. In: N. G. CARR & B. A. WHITTON (eds.), *Botanical Monographs* **9**: 186–200. Blackwell Scientific Publications, Oxford-London-Edinburgh-Melbourne.

LEGAULT-DEMARE, J., B. DESSAUX, T. HEYMAN, S. SEROR & G. P. RESS (1967): Studies on hybrid molecules of nucleic acids. I. DNA-DNA hybrids on nitrocellulose filters. *Biochem. Biophys. Res. Commun.* **28** (4): 550–557.

LEY, J. DE & R. TIJTGAT (1970): Evaluation of membrane filter methods for DNA-DNA hybridization. *Antonie van Leeuwenhoek* **36**: 461–474.

MARMUR, J. (1961): A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**: 208–218.

NICOLS, H. W. (1973): Growth media – freshwater. In: J. R. STEIN (ed.) *Handbook of phycological methods*, Culture methods and growth measurements: 8–23. Cambridge University Press.

SAFFERMAN, R. S. & M. F. MORRIS (1963): Algal virus: Isolation. *Science* **140** (3567): 679–680.

— (1964): Control of algae with viruses. *J. Amer. Water Works Ass.* **56** (9): 1217–1225.

SCHILDKRAUT, C. L., J. MARMUR & P. DOTY (1962): Determination of base composition of deoxyribonucleic acid from its buoyant density in CsCl. *J. Mol. Biol.* **4**: 430–434.

SHESTAKOV, S. V. & N. T. KHYEN (1970): Evidence for genetic transformation in blue-green alga Anacystis nidulans. *Mol. gen. Genetics* **107**: 372–375.

STAM, W. T. & H. C. HOLLEMAN (1975): The influence of different salinities on growth and morphological variability of a number of Phormidium strains (Cyanophyceae) in culture. *Acta Bot. Neerl.* **25** (5–6): 379–390.

STANIER, R. Y., R. KUNISAWA, M. MANDEL & G. COHEN-BAZIRE (1971): Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriol. Rev.* **35** (2): 171–205.

STARR, R. C. (1964): The culture collection of algae at Indiana University. *Amer. J. Bot.* **51** (9): 1013–1044.

VANCE, B. D. (1966): Sensitivity of Microcystis aeruginosa and other blue-green algae and associated bacteria to selected antibiotics. *J. Phycol.* **2**: 125–128.

VENEMA, G., R. H. PRITCHARD & T. VENEMA-SCHRODER (1965): Fate of transforming deoxyribonucleic acid in Bacillus subtilis. *J. Bacteriol.* **89**: 1250–1255.

WIEDEMAN, V. E., P. L. WALNE & F. R. TRAINOR (1964): A new technique for obtaining axenic cultures of algae. *Can. J. Bot.* **42**: 958–959.