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Comparison Between the Symbiotic *Nostoc* of the Lichen *Nephroma laevigatum* Ach. and its Cultured, Isolated *Nostoc* by Recombinant DNA

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Abstract

Resynthesis of many plant-cyanobacteria symbiotic associations from their isolated components is very difficult and often unsuccessful, particularly with lichens, that are considered a highly selective association. Therefore, uncertainty has repeatedly been expressed as to the reliability of reports concerning the use of cultured symbiont isolates as long as Koch's postulate has not been satisfied. In order to prove identity or diversity of the symbiotic *Nostoc* of the lichen *Nephroma laevigatum* and its cultured isolates, we compared DNA extracted from the intact *Nostoc* cyanobiont, from cultured isolates and from non-symbiotic *Nostoc* strains. Endonucleases (*Eco*RI, *Hind*III and *Msp*I) digested DNA was blotted (Southern) and hybridized to labelled *nif* and *rbc*L gene probes from the free-living *Anabaena* sp. PCC 7120. A total of 11 enzyme-probe combinations revealed identical and variable hybridization patterns. The hybridization patterns of the non-symbiotic *Nostoc* strains differed greatly from those of the intact cyanobiont and the cultured isolates. Possibilities that may account for the pattern differences are suggested.

Keywords: *Nostoc*, *Nephroma laevigatum*, recombinant DNA

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1. Introduction

Nephroma laevigatum is a stratified (heteromerous) N_2-fixing lichen containing *Nostoc* sp. as its photobiont. *N. laevigatum* produces numerous fruiting bodies containing an enormous number of ascospores (Galun, 1970).

The cyanobacteria-fungus symbiosis of N_2-fixing lichens is assumed to be a highly specific association (Stewart et al., 1983). Consequently, selection of compatible partners in nature is essential for the establishment of new specimens. Reliable studies on this selection process would require isolation of the components and their reconstruction under controlled conditions. This procedure would acertain the origin of the isolates, exclude the possibility that contaminants are involved and would enable testing the level of specificity of interaction. Since, however, resynthesis of lichens from their separated and cultured components is, if successful, a protracted process with very limited yields (Galun, 1988), a different approach has to be employed (Bubrick and Galun, 1980; Bubrick et al., 1985).

Upon isolation and culturing the symbionts regain their free-living life style (Marton and Galun, 1976; Bubrick, 1988; Kardish et al., 1989). The surface properties of these isolates are of interest when studying the range of biont combinations and mechanism of discrimination between potential and foreign partners.

Prior to such studies it is essential to acertain homology between the cultured isolate and its symbiotic counterpart. Because of the difficulty encountered in providing proof for homology by reconstructing the lichen from separated bionts we tested whether by comparing specific regions of DNA of the intact cyanobiont (*Nostoc* sp.) of the lichen *N. laevigatum* and its presumed cultured isolates, we can solve this problem. For this purpose we used probes from the free-living *Anabaena* sp. PCC 7210 and compared restriction sites of *nif* and *rbc*L gene regions of the cultured *Nostoc* isolated from the lichen *N. laevigatum* to those of the *Nostoc* in the intact lichen thallus and to some non-symbiotic *Nostoc* strains.

2. Materials and Methods

Organisms

Nephroma laevigatum Ach. was collected from bark of oak trees at Har Meron (Upper Galilee, Israel). *Nostoc* sp. was isolated from a free-living colony growing on soil at the same habitat. *Nostoc muscorum* 7119 was obtained

from E. Tel-Or, Faculty of Agriculture, Hebrew University, *Nostoc muscorum* 1453–12b was provided by the Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen.

Isolation of the symbiotic Nostoc

Nostoc was isolated from *N. laevigatum* by two different techniques, from lichen material collected at three different seasons:

1. Thallus fragments were washed for 1 hr with running tap water, then macerated delicately with mortar and pestle, filtered through a nylon screen (20 mesh) and plated on BG-11 medium (Stanier et al., 1971), solidified with 1.2% agar in 9 cm Petri dishes. On top of this layer, additional $\sim$ 3 ml of the same medium, warmed to $\sim$ 50°C, were poured. Most contaminating organisms developed inside the medium (between the two layers) whereas the *Nostoc* filaments grew through the upper layer. After several transfers to the same medium, the enriched cultures served as inoculi of liquid BG-11 medium.
2. The medulla was removed manually from dry thallus fragments, the "enriched cyanobiont layer" was washed as above and small fragments were inoculated on a medium consisting of fine silica gel powder mixed with a modified Bristol liquid nutrient solution (1:2 w/v), as described by Galun et al. (1972). *Nostoc* filaments, which grew out from the inoculi, after $\sim$ 4 months, were transferred to agarized BG-11 medium as above. After further purification by repeated restreaking on the same medium, or by the "double layer technique" as above, cultures were transferred to liquid BG-11 medium.

   Cultures were maintained at 21 ± 1°C and exposed to fluorescent "white" illumination (6.5 $J.m^{-2}sec^{-1}$; 16 hr light and 8 hr dark). Liquid cultures were shaken on a gyratory shaker (New Brunswick, NJ, USA).

DNA extraction

DNA was extracted from a,the intact lichen thallus; b, enriched *Nostoc* layer (by manual removal of the fungal medulla); c, enriched mycobiont layer (the above medulla, that in some cases contained a few *Nostoc* cells); d, e, and f, cultured *Nostoc* isolates; g, h, and i, cultured non-symbiotic strains.

DNA extraction was performed according to Breiman et al. (1987) with minor modification. One g (f.w.) of *Nostoc* cells or 1 g (f.w.) of *N. laevigatum* thalli were crushed to fine powder with mortar and pestle in liquid nitrogen in the presence of a small quantity of acid-washed sand. The powder was mixed with 3.5 ml DNA isolation buffer (0.1 M NaCl; 0.1 M Tris-HCl; pH 8.5; 0.5 M EDTA; 0.2% SDS; 0.1 mg/ml proteinase K). The homogenate was extracted once with phenol-chloroform (1:1 v/v) and once with chloroform-isoamyl alcohol (24:1) and the aqueous phase was ethanol precipitated in the presence of 0.2 M sodium chloride. The DNA was redissolved in 1.5 ml of TE buffer (0.01 M Tris-HCl, pH 8.0; 0.001 M EDTA) and incubated for 30 min at 37°C with 100 mg/ml RNAse A and 60 units/ml RNAse T1. The aqueous phase was reextracted with phenol-chloroform (1:1 v/v) and chloroform-isoamyl alcohol (24:1). The DNA was ethanol precipitated in the presence of 2 M ammonium-acetate and the precipitate was vacuum-dried (Speed Vac concentrator, Instr. Inc., NY) and resuspended in 200 μl TE buffer. The DNA extracted from *N. laevigatum* was further purified by mounting it on a $CsCl_2$ gradient of 1.5 g/cm^2 and then centrifuged for 48 hr at 35,000 rpm (rotor Ty-65, Beckman Ultracentrifuge) at 18°C.

Restriction endonuclease digestion

Approximately 5 μg extracted DNA were digested with 10 units/μg DNA of *Eco*RI, *Hind*III or *Msp*I respectively, (purchased from IBI, New Haven, CT) in the buffer recommended by the supplier. Digestion reaction was carried out at 37°C for 18 hr.

Agarose gel electrophoresis and blotting

The digested DNA was run on 0.8% horizontal agarose 15 cm gels. Electrophoresis was performed overnight at 40 volts in TBE buffer (0.089 M Tris; 0.089 M boric acid; 0.002 M EDTA, pH 8.0). Lambda phage DNA digested with *Hind*III were used as molecular weight standards. After electrophoresis, the gel was photographed under UV light (310 nm), and denatured in 0.2 M NaOH, 0.6 M NaCl for 1 hr.

Hybridization procedures

The separated DNA was transferred to nylon filters (GeneScreen Plus, NEN Research Products) according to Southern (1975). The filters were baked for 2 hr at 80°C and prehybridized for 6 hr in a plastic bag at 65°C, according to the manufacturer's instructions. For hybridization a ^{32}P labelled probe

and 100 μg/ml sheared salmon sperm DNA were injected into the plastic bag and incubated for at least 16 hr. The probes were removed according to the manufacturer's instructions for reuse of the membranes.

Recombinant DNA clone and ^{32}P labelling

The following pAn plasmids carrying *Anabaena* PCC 7120 chromosomal fragments were used as radiolabelled probes: pAn 154.3, containing *nif*H and the 5′ region of *nif*D (1.8 kb); pAn 207.8, containing an internal *nif*K fragment (0.8 kb); pAn 207.3, containing an insert (1.8 kb) from the 11 kb DNA segment of *Anabaena* PCC 7120 located inside *nif*D and is excised during heterocyst differentiation (Rice et al., 1982; Golden et al., 1985) and pAn H27 including a 1.9 kb *rbc*L fragment.

The probes were labelled by nick translation reagent kit (BRL — Bethesda Res. Lab.) according to Rigby et al., 1977). The specific activity of the labelled DNA was $\sim 10^8$ cmp/μg DNA.

3. Results

Total DNA was extracted from *N. laevigatum* thalli as well as from its cultured *Nostoc* isolates and from non-symbiotic *Nostoc* strains. The DNAs were

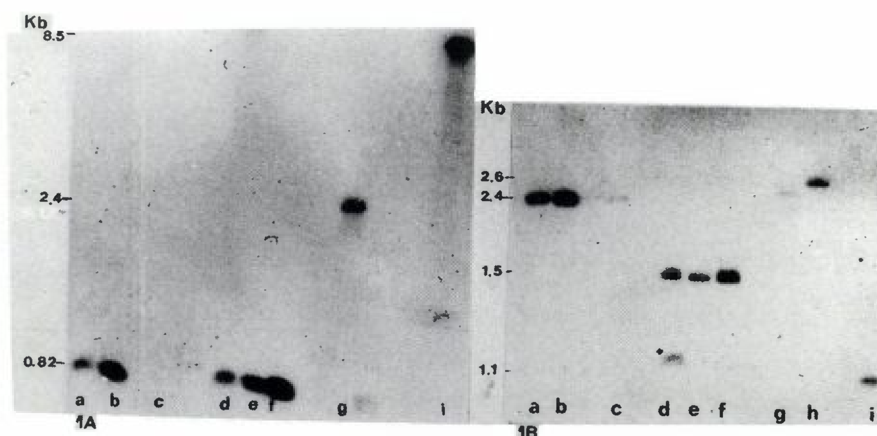

Figure 1. Southern blot hybridization of total DNA with the *nif*K gene. A = digested with *Eco*RI; B = digested with *Hind*III; a = the intact lichen thallus; b = enriched *Nostoc* layer; c = enriched mycobiont layer; d,e,f = three cultured *Nostoc* isolates; g,h,i = cultured non-symbiotic *Nostoc* strains.

digested with *Eco*RI, *Hind*III or *Msp*I. The digested DNA was separated on agarose gels, blotted onto nylon filters and hybridized with ^{32}P-labelled probes.

Identical hybridization patterns of *Eco*RI digested DNA extracted from the lichen thallus and from the cultured, symbiotic *Nostoc* isolates were obtained with the *nif*K probe (Fig. 1A). A 0.82 kb band appeared in both DNA extracts. The hybridization patterns of the non-symbiotic *Nostoc* strains with this probe revealed different hybridization bands of 2.4 kb and 8.5 kb (Fig. 1A). However, when the DNAs were digested with *Hind*III and hybridized with the same probe (*nif*K) different patterns were obtained. While DNA extracted from the intact lichen revealed a 2.4 kb band, a 1.5 kb band appeared in the three patterns of the symbiotic cultured *Nostoc* isolates (Fig. 1B). As was the case in the *Eco*RI digests, different hybridization patterns were obtained from the extracts of the non-symbiotic *Nostoc* strains (Fig. 1B). An additional band

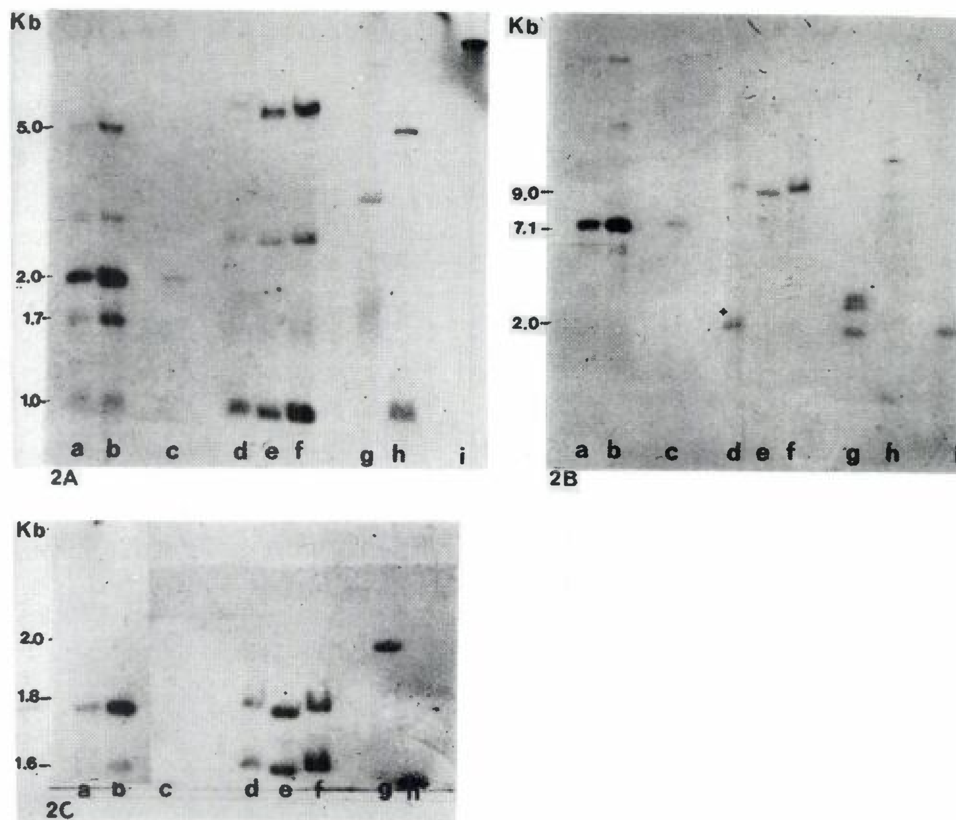

Figure 2. Southern blot hybridization of total DNA with the *nif*H gene. A = digested with *Eco*RI; B = digested with *Hind*III; C = digested with *Msp*I; a–i see Fig. 1.

(1.1 kb) was observed in the pattern of one of the isolates (Fig. 1B, lane d). This was due to contamination in the culture of one of the symbiotic cultured *Nostoc* isolates by the non-symbiotic strain designated i.

The *nif*H probe displayed a more complex picture. The *Eco*RI digest revealed 5 hybridization bands in the lichen's DNA preparation. These bands ranged in size from 5.0 kb to 1.0 kb. Three of these bands (of 1.0 kb, 3.1 kb and 5 kb) are in accordance with the three bands of the symbiotic cultured *Nostoc* isolates patterns (Fig. 2A). In the *Hind*III digest only one band (9.0 kb) of the symbiotic cultured *Nostocs*' DNA hybridized to the *nif*H probe (Fig. 2B). This band, however, differs from the 3 bands observed in the DNA extracted from the intact thallus (Fig. 2B). In the *Msp*I digest of DNA extracted from the lichen and the symbiotic cultured *Nostoc* isolates, two common bands of

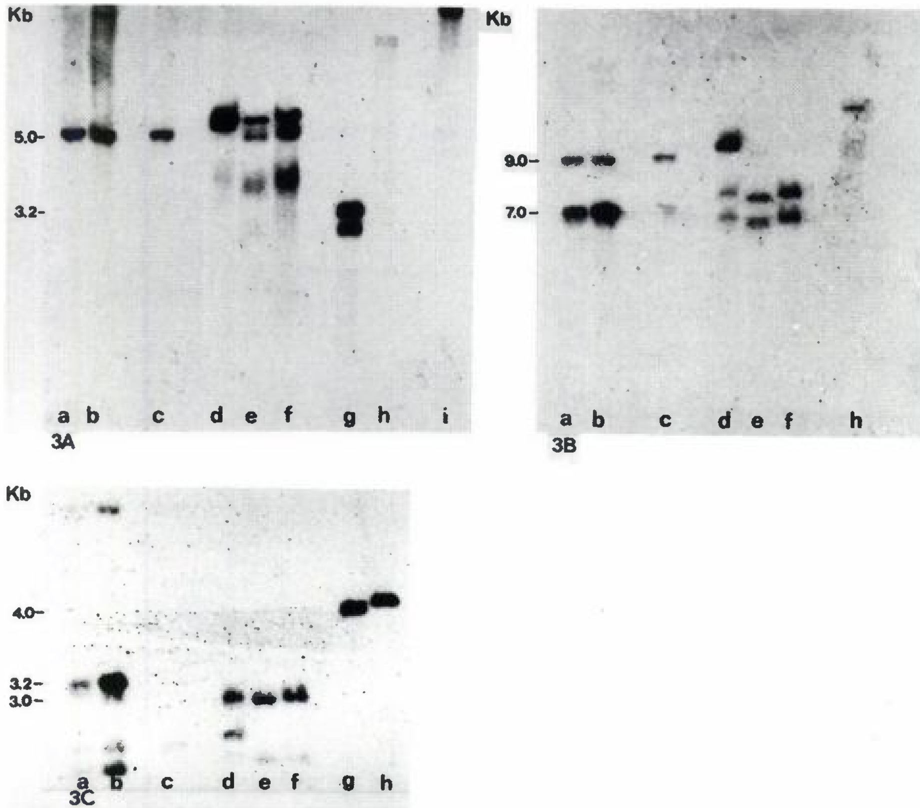

Figure 3. Southern blot hybridization of total DNA with the *rbc*L gene. A = digested with *Eco*RI; B = digested with *Hind*III; C = digested with *Msp*I; a–i see Fig. 1.

Table 1. Identify and variability of hybridization bands of DNA extracted from *Nostoc* of the intact lichen *N. laevigatum* and three cultured isolates using 11 enzyme-probe combinations.

Endonucleases	Probes											
	*nif*K			*nif*H			pAn207.3			*rbc*L		
	total	ident-tical	variable	total	iden-tical	variable	total	iden-tical	variable	total	ident-tical	variable
*Eco*RI	2	2	0	8	6	2	1	1	0	3	2	1
*Hind*III	2	0	2	4	0	4	n.d.	n.d.	n.d.	5	4	1
*Msp*I	n.t.	n.t.	n.t.	4	4	0	n.d.	n.d.	n.d.	7	4	3

n.t. = not tested; 1–8 = number of bands
n.d. = not detected.

1.6 kb and 1.8 kb were observed (Fig. 2C). The pattern of the 3 non-symbiotic *Nostoc* strains was completely different in all cases.

One common band, 3.9 kb, was observed both in the intact *Nostoc* and the 3 symbiotic cultured *Nostoc* isolates, when DNA digested with *Eco*RI was hybridized with the 1.8 kb fragment of pAN 207.3 which is excised during heterocyst formation in *Anabaena* PCC 7120 (date not shown). No hybridization bands could be detected with *Hind*III and *Msp*I digests.

In each of the three digests used, the lichen and the symbiotic cultured *Nostoc* isolates' DNA revealed a common band homologous to the *rbc*L probe: 5.0 kb, 7.0 kb and 3.2 kb with *Eco*RI, *Hind*III and *Msp*I respectively (Figs. 3A, 3B, 3C), an additional common band of 1.0 kb with *Msp*I (Fig. 3C) and several variable bands. The patterns of the 3 non-symbiotic *Nostoc* strains were again completely different.

The information obtained from all the hybridization patterns is summarized in Table 1.

4. Discussion

A comparison was made between DNA extracted from the intact lichen *N. laevigatum*, from three cultures of *Nostoc* isolated from the lichen and three non-symbiotic *Nostoc* strains. A total of 11 enzyme-probe combinations were used. The analyses performed revealed a total of 23 common bands and 13 variable bands (Table 1).

The hybridization patterns revealed by the non-symbiotic *Nostoc* strains differed greatly from those obtained from the lichen thallus and the cultured cyanobiont isolates. The enriched mycobiont fraction, obtained by manual removal of the fungal medulla, contained in some cases cyanobiont cells as the result of incomplete separation. Whenever such *Nostoc* cells were present, this fraction also gave rise to hybridization bands (lane c). Whenever the separation was complete, DNA extracted from the enriched mycobiont layer did not hybridize with the probes specific to the N_2-fixing, photosynthesizing cyanobiont, as was expected.

The hybridization patterns obtained may suggest that we have isolated and cultured a contaminating N_2-fixing, photosynthetic microorganism and not the native *Nostoc* of *N. laevigatum*. Since, however, the hybridization patterns of 3 cultured isolates which have been prepared by 3 different persons from lichen samples collected each at a different season and by different isolation methods are identical, such a possibility is very unlikely. We therefore presume one of the following possibilities: (1) The discrepant patterns of DNA fragments

from the two populations (symbiotic and cultured) can be attributed to a phenomenon similar to the somaclonal variations in higher plants known from tissue culture cycles involving a disorganized callus phase (Karp and Bright, 1985). It has to be emphasized that during changing its status from symbiotic to cultured, the *Nostoc* of *N. laevigatum* undergoes striking morphological (Kardish et al., 1989) and physiological (Bubrick, 1988) modifications. Such modifications in the reverse direction occur during integration of the symbionts and the formation of a lichen thallus bearing no resemblance to its free-living components. (2) It is possible that an originally variable *Nostoc* population exists in the lichen thallus and that one type predominates, which becomes overwhelmed by its counterpart during the dramatic stress the isolate has to cope with when transferred to culture. Here again analogy can be drawn from tissue culture induced single gene variants among progeny of regenerated plants (Lee and Phillips, 1988). (3) Rearrangement of the *nif* gene region occurs in *Anabaena* when a vegetative cell differentiates into a heterocyst (Golden et al., 1985). There may be rearrangement during readaptation of the symbiotic *Nostoc* to the free-living growth-form, which is accompanied by very dramatic modifications (Kardish et al., 1989).

Since the *Eco*RI and the *Msp*I (a 4 bp cutter) hybridization patterns with the *nif*H probe, are identical in the DNA extracted from the lichen and the cultured isolates, we may assume homology between internal regions of the *nif* structural genes which are not subjected to rearrangement events. RFLP analyses of two *nif*K genes and four *nif*H genes from different N_2-fixing organisms (computer sequence analysis) did not display any similarity of restriction fragments. According to the hybridization pattern resulting from *nif*H probe and *Eco*RI digestion, it seems that having two cyanobionts is a feasible possibility, however, no additional support could be found for this assumption in other hybridization patterns. Moreover, it does not contradict the hypothesis on rearrangement caused by physical shock.

Further DNA sequence analyses will perhaps clarify this complex situation.

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