Field investigations on cyanobacterial specificity in *Peltigera aphthosa*

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Summary

• Possibilities of introducing new cyanobacterial strains into established lichen symbioses were examined by manipulating thalli of *Peltigera aphthosa* and by using the trnL (UAA) intron to identify specific cyanobacterial strains.

• *P. aphthosa* has *Nostoc* symbionts in cephalodia on the upper surface of the thallus. In three different *P. aphthosa* populations, each consisting of seven thalli, all cephalodia were experimentally removed. The manipulated lichens were then inoculated with known strains of cultured cyanobacteria and left to develop new cephalodia. After a summer in the field the lichens were harvested and the strain identities of cyanobacterial symbionts in 80 newly formed cephalodia were determined. All epiphytic colonies of free-living cyanobacteria found were also analysed.

• Foreign cyanobacteria were not readily incorporated into established *P. aphthosa* thalli. All newly formed cephalodia contained the same intron sequence, which was identical to that found in the removed cephalodia. At least two inoculated *Nostoc* strains were able to survive as epiphytic colonies on experimental thalli. Both strains had originally been isolated from bipartite *Peltigera* species.

• Results indicate that associations between cyanobacteria and lichen-forming fungi can be very specific and stable, which contrasts with the general view that cyanobacterial symbioses are rather unspecific.

Key words: *Peltigera aphthosa*, *Nostoc*, cephalodia, lichen, cyanobacteria.

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Introduction

Cyanobacteria belonging to the genus *Nostoc* are capable of forming symbioses with many different plants and fungi (Meeks, 1998; Adams, 2000; Rai *et al.*, 2000). Most symbiotic associations with plants, with the *Azolla*-association as an exception, appear to be relatively nonspecific in the sense that several different *Nostoc* strains can infect individual plant species and specific *Nostoc* strains can enter into symbioses with several types of host plant. For example, in reinfection studies with thalli of axenically grown *Anthoceros*, free-living *Nostoc* isolates as well as isolates from symbiotic associations with bryophytes, cycads, lichens, and the angiosperm *Gunnera* have been able to enter into symbiosis with the hornwort (Enderlin & Meeks, 1983). Similar results have been obtained from studies with axenically germinated *Gunnera* seedlings (Bergman *et al.*, 1992). Thus, there seems to be a wide range of *Nostoc* strains capable of establishing symbioses with different host plants. The physiological characteristics required for establishing symbiosis are not known, but motile hormogonia and the capacity to develop heterocysts are thought to be key factors (Adams, 2000). Hormogonia are short cyanobacterial filaments that represent the motile and infective stage in the *Nostoc* lifecycle whilst heterocysts are specialized cells responsible for N_2 fixation.

We have previously analysed cyanobacterial diversity in several species of *Peltigera* and *Nephroma*, lichen-forming ascomycetes with *Nostoc* cyanobionts (Tschermak-Woess, 1988). Interestingly, these lichen symbioses exhibit much higher levels of specificity than do plant symbioses. Generally, only one or two *Nostoc* strains have been found to be associated with each lichen species and, when several samples from different parts of a single lichen thallus have been examined, almost invariably only one *Nostoc* strain has been found.

Consistent patterns of specificity have been observed over a wide range of geographical scales, and the strain identities of *Nostoc* cyanobionts have always been more restricted by the species identities of lichens than by the geographical origins of the samples. This has been shown within single collecting sites, between different localities in central Sweden, between collecting sites in Sweden and Finland, and between collecting sites in Sweden and the USA (Paulsrud & Lindblad, 1998; Paulsrud *et al.*, 1998; Paulsrud *et al.*, 2000). How these patterns should be interpreted is not quite clear, but one feasible explanation is that, under natural conditions, the establishment of new lichens through *de novo* lichenization from fungal spores and free-living cyanobacteria is rare. Instead the lichens we have studied may mainly disperse by thallus fragments and other diaspores containing both bionts, thus leading to the observed patterns in specificity. In any case, some *Nostoc* strains are clearly capable of forming symbioses with more than one species of lichen-forming fungi. One example of this was found in samples of *Peltigera neopolydatyla* and *P. aphthosa* which contained cyanobionts with identical trnL (UAA) intron sequences (Paulsrud *et al.*, 1998). Conversely, some lichen-forming fungi seem to be capable of forming symbioses with more than one strain of *Nostoc*, as was seen in the case of *P. aphthosa* and some other *Peltigera* species (Paulsrud & Lindblad, 1998; Paulsrud *et al.*, 1998; Paulsrud *et al.*, 2000).

In the present study we have examined the possibility of introducing new cyanobacterial strains into established lichen thalli in the field. We are not aware of any previous studies in which cyanobacterial specificity in intact lichens has been investigated experimentally. This is probably because cyanolichens are notoriously difficult to cultivate under laboratory conditions (Ahmadjian, 1993; Yoshimura *et al.*, 1994; Stöcker-Wörgötter, 1995). For this reason, we decided to perform inoculation experiments with well-established lichens in their natural habitat. The species used was *Peltigera aphthosa*, a tripartite lichen, which has a green alga, *Coccomyxa*, as its primary photobiont and *Nostoc* as its secondary photobiont. In tripartite lichens the green algae generally produce most of the photosynthate whilst the cyanobacteria function mainly in N_2 fixation (Rai, 1988). In *P. aphthosa* the cyanobacterial symbiont is confined to specialized structures on the upper surface of the thallus. These cephalodia consist of *Nostoc* colonies surrounded by dense fungal tissue. As the cephalodia represent only a small portion of the thallus surface, and because photosynthate is mainly produced by the green algae, cephalodia of *P. aphthosa* can be experimentally removed without killing the lichen (Sundberg *et al.*, 1999). This will cause the lichen to lose its ability to fix N_2 with possible nitrogen starvation as a consequence, but under favourable growth conditions new cephalodia will appear on the thallus surface within a few weeks. We predicted that this phenomenon might open possibilities for introducing new *Nostoc* strains into an established lichen symbiosis. Theoretically, artificial inoculations could even lead to patterns of cyanobacterial diversity similar to those that have been observed in *P. venosa*, another tripartite *Peltigera* species, where different *Nostoc* colonies from single thalli have different intron sequences (Paulsrud *et al.*, 2000). In our previous studies we have found that all *P. aphthosa* thalli collected from Finland and Sweden have invariably housed only one of two *Nostoc* strains in all of their cephalodia (Paulsrud & Lindblad, 1998; Paulsrud *et al.*, 1998). A single *Nostoc* strain is also capable of forming both morphotypes of this lichen, as identical intron sequences were found in both bi- and tripartite thallus sections of a *P. aphthosa* photosymbiodeme (Paulsrud *et al.*, 1998).

Materials and Methods

Isolation and identification of lichen-forming cyanobacteria

The lichen-forming cyanobacteria that were used in the inoculation experiment were isolated by culturing on agar plates. Lichen thalli were rinsed in water and small fragments of the cyanobacterial zone were carefully removed from the lichen thallus using a sterile scalpel. The excised pieces were placed on agar plates ($Bg11_0$ medium containing 0.8% w/v agarose) and left at room temperature. Growth was followed by examination of tissue using a dissecting microscope to ensure that it was the cyanobacterium from the cephalodium or cyanobacterial zone of the lichen that gave rise to the culture. After a few weeks to months, inocula from growing cyanobacterial colonies were transferred to new plates. The subcultures were started by taking hormogonia (for *Nostoc* strains with hormogonia, Fig. 1a) or single packets of cyanobacteria (for *Nostoc* strains not forming hormogonia, Fig. 1b) from the margins of the original colonies.

The strain identities of all cyanobacterial cultures were analysed by sequencing the trnL (UAA) intron. The intron was amplified using nested PCR and primers A/C (outer primers) as described in Paulsrud & Lindblad (1998) and primers TL25/TL23 (inner primers) as described by Paquin *et al.* (1997). Amplified fragments were purified using PCR Wizard kit (Promega, Madison, WI, USA) and 20 ng of purified product were used in a 5-μl sequencing reaction with Big dye chemistry (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. The intron sequence from the cultured strain was compared with the intron sequence obtained from the intact lichen in order to verify that the correct cyanobacterium had been isolated. In total, five different lichen-forming *Nostoc* strains were used. The strains originated from the lichens *Peltigera aphthosa* (two different *Nostoc* strains from different *P. aphthosa* populations), *P. canina*, *P. membranacea*, and *Nephroma resupinatum* (Table 1). In addition to the isolated lichen cyanobionts, two laboratory strains were included; *N. punctiforme* PCC 73102 which can form symbiotic associations with several hosts and has thus become a model strain in studies of cyanobacterial symbioses

Table 1 *Nostoc* strains isolated from lichens in the present study. GenBank entry refers to the sequence of the trnL (UAA) intron reported earlier (Paulsrud & Lindblad, 1998; Paulsrud *et al.*, 1998, 2000). Colony morphology refers to appearance of the strain when grown on B_gII_o agar plates. Motility is shown as either + (produces hormogonia under the growth conditions used in these experiments) or – (does not produce hormogonia). Growth rate is listed as either slow (doubling time in the range of weeks) or fast (doubling time in the range of days) on agar plates

Strain	Lichen host	Genbank	Colony	Motility	Growth rate
Nostoc-Pm	*Peltigera membranacea* (Ach.) Nyl.	AF176605	variable*	+	fast
Nostoc-Pc	*Peltigera canina* (L.) Willd.	AF019921	mat-like	+	fast
*Nostoc-Pa*1	*Peltigera aphthosa* (L.) Willd.	AF019915	pearl-like	–	slow
*Nostoc-Pa*2	*Peltigera aphthosa* (L.) Willd.	AF055656	pearl-like	–	slow
Nostoc-Nr	*Nephroma resupinatum* (L.) Ach.	AF055660	mat-like	+	fast

*This strain may form both small elevated undulate colonies and a mat-like growth when hormogonia formation is induced.

(Lindblad *et al.*, 1991; Jansson, 1997; Meeks *et al.*, 1999), and *Anabaena (Nostoc)* PCC 7120 which is a well characterized laboratory strain unable to enter into symbiotic associations.

Inoculation experiments

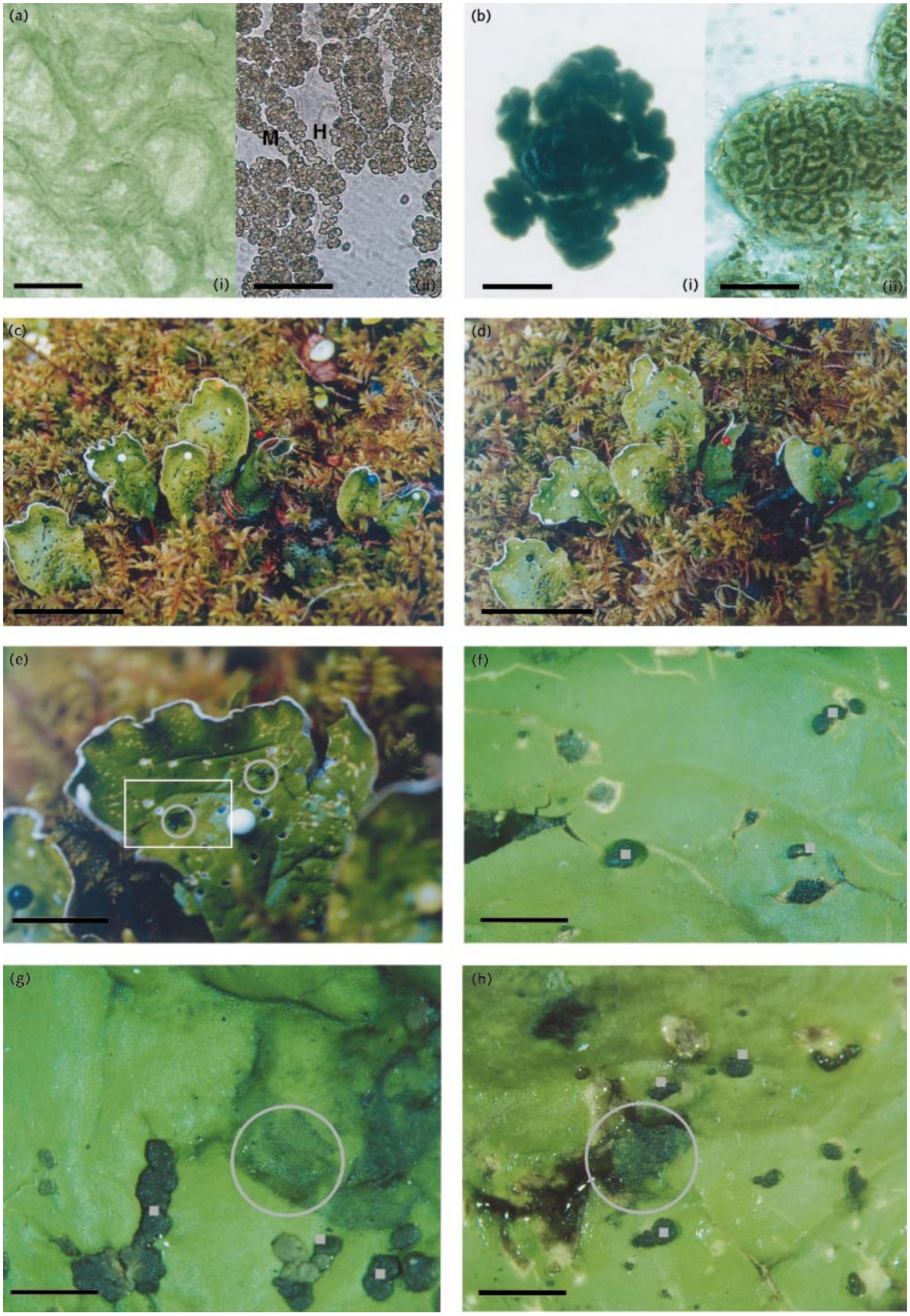
The site chosen for the inoculation experiment is located at Näcksjöberget in Särna, western Sweden (site 1 in Paulsrud & Lindblad, 1998). The experimental site is situated at the base of a steep cliff and the lichens grow on moss-covered boulders and logs. The site is shaded by spruce trees and is rather humid. Thalli of *Peltigera aphthosa* were manipulated *in situ* by removing all cephalodia using a sterile scalpel (compare Fig. 1c,d). Three different lichen populations less than 100 m apart, each consisting of seven thalli, were treated in this way and examined with a hand lens before they were inoculated with cultured *Nostoc*. The inoculum was added as liquid culture. The different cultured strains were suspended in $Bg11_0$ medium and applied over the lichen thalli using a sterile pipette. All thalli were completely soaked by the inoculum but, in some cases, the deposition of cyanobacteria may have been rather uneven, as the cyanobacteria of some strains tended to clump together. This is especially true for the two *Nostoc* strains from *P. aphthosa* (both resembling strain *Nostoc-Pa*1 in Fig. 1b) which produced very compact, hard colonies that were difficult to break down and distribute evenly over a lichen surface. After inoculation, at least three deposits of *Nostoc*-inoculum could be seen on each thallus (for one example, see Fig. 1e). In total, seven different *Nostoc* isolates were used for inoculations, each strain on a single manipulated *Peltigera* thallus in each of the three populations.

The *Peltigera* thalli were manipulated in early summer (June 4, 2000) and harvested for analysis in early autumn (September 23, 2000). The lichens were clearly hydrated during the inoculation. There was some rainfall at the experimental site the day before and after, but not during the day of inoculation. In September, 17 out of 21 manipulated *Peltigera* thalli were recovered. The remaining four thalli were rejected because they were morbid and/or had become overgrown by terrestrial bryophytes. At least two thalli that had been inoculated with each of the 7 *Nostoc* strains could be recovered. The manipulated thalli had grown during the summer and numerous new cephalodia had developed on their upper surfaces, especially in the marginal parts. New cephalodia had developed both on scars left by removed cephalodia and on untouched cortical surfaces between the scars. However, on some thalli, areas without new cephalodia also occurred and only scars of removed cephalodia were seen.

The harvested lichens were taken to the laboratory where the strain identities of the cyanobacterial symbionts in several cephalodia from each thallus were determined by sequencing of the cyanobacterial trnL (UAA) intron as described above. Under the dissecting microscope, colonies of free-living cyanobacteria were detected on the upper surfaces of some lichen thalli, and these too were analysed. When choosing samples for analysis, special attention was given to cephalodia that had developed on thallus sections where large deposits of cyanobacterial inoculum had accumulated during inoculation (see Fig. 1e,f), as well as to those cephalodia that were in close contact with free-living, epiphytic cyanobacteria on the thallus surface (see Fig. 1g,h). In total, trnL (UAA) intron sequences from 80 cephalodia and seven free-living *Nostoc* colonies were obtained from the manipulated thalli after the field experiment.

Results

Before the inoculation experiment, five different strains of lichen-forming *Nostoc* were brought into culture. Sequencing of the trnL (UAA) intron confirmed that all cultured strains were the same as those found in the corresponding lichen thalli. During isolation, the cyanobacteria from *Peltigera membranacea* (*Nostoc-Pm*), *P. canina* (*Nostoc-Pc*) and *Nephroma resupinatum* (*Nostoc-Nr*), started to grow out of lichen fragments within approx. 1 wk, but for the two strains from *Peltigera aphthosa*, growth was much slower (taking months). As expected, all cyanobacterial strains isolated from the lichens were of the *Nostoc*-type. The cyanobacteria were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filamentous, heterocystous, they produced isopolar trichomes with no evidence of branching or meristematic zones, and their cells were more or less spherical. The typical life-cycle of *Nostoc*, with morphologically distinct hormogonia and with vegetative filaments exhibiting different degrees of coiling (Rippka *et al.*, 1979; Mollenhauer, 1988), was seen in strains *Nostoc-Pm*, *-Pc* and *-Nr*. The two strains isolated from *Peltigera aphthosa* (*Nostoc-Pa*1, *-Pa*2) did not develop hormogonia under any conditions tested. These strains thus lacked motility and were slow to spread on agarose plates. The cyanobionts of *P. aphthosa* tended to grow as small pearl-like colonies (about 1 mm in diameter) eventually giving rise to grape-like clusters (see Fig. 1b). The same morphology was maintained when the strains were grown in liquid culture.

Analysis of the trnL (UAA) intron from cephalodia from the experimental site before the inoculations showed that they all contained the same intron sequence (GenBank accession number AF019915, see *Nostoc-Pa*1 in Table 1). This sequence had also been obtained from *P. aphthosa* samples collected from the same field site 5 yr before the present study (Paulsrud & Lindblad, 1998). After inoculation and subsequent growth, 80 newly formed cephalodia were analysed. All of these contained identical intron sequences, this sequence also being identical to those obtained from excised cephalodia before inoculation. Thus, the original *Nostoc* strain turned up in all new cephalodia on all manipulated thalli, despite massive inoculations of six other *Nostoc* strains. In addition to cyanobacteria in cephalodia, seven colonies of free-living *Nostoc*s were found growing on four different *P. aphthosa* thalli. Two different intron sequences were identified among the free-living colonies, corresponding to the foreign *Nostoc* strains that had been introduced onto these thalli (Fig. 1g,h). Thus, all three thalli that had been inoculated with *Nostoc-Pc* had this specific strain as an epiphyte on their upper surface. Concurrently, one of the two recovered thalli that had been inoculated with *Nostoc-Pm* had this strain as an epiphyte. None of the other *Nostoc* strains were detected from the manipulated lichens.

Discussion

Clearly, foreign cyanobacteria are not readily incorporated into established thalli of *Peltigera aphthosa*. All of the 80 newly formed cephalodia that were analysed contained the same intron sequence. This sequence was also identical to that found in the original cephalodia which were removed before the experiment. This result is quite significant as all except one of the seven cyanobacterial strains used for inoculation had been isolated from symbiotic systems, five from different cyanolichens and one from the coralloid roots of a cycad. The last mentioned strain (*Nostoc punctiforme* PCC 73102) had previously shown broad symbiotic competence with several types of host plants (Enderlin & Meeks, 1983; Bergman *et al.*, 1992). The field site was the same as in a previous study (Paulsrud & Lindblad, 1998), so we were also able to confirm that the original *Nostoc* strain had been present in the *P. aphthosa* population for at least 5 yr before the experiment. Both the fact that we were unable to introduce foreign *Nostoc* strains into established lichen thalli and the apparent lack of temporal variation in cyanobiont composition are fully consistent with our previous findings of little or no spatial variation in the cyanobacterial symbionts of specific lichen species (Paulsrud *et al.*, 1998, 2000).

There was substantial variation in the phenotypic characteristics of our cyanobacterial isolates (Table 1, Fig. 1a, Fig. 1b). There is some controversy over the generic delimitation of *Nostoc* and several classification schemes with slightly different taxonomic concepts are currently in use (Geitler, 1932; Rippka *et al.*, 1979; Castenholz, 1989; Komárek & Anagnostidis, 1989). The production of hormogonia is an important diagnostic feature for *Nostoc*, *sensu* Rippka (Rippka *et al.*, 1979). In our material the two isolates from *Peltigera aphthosa* (*Nostoc-Pa*1, *-Pa*2) did not develop hormogonia under any

Fig. 1 (a,b) Lichen-forming *Nostoc* strains in culture; colony morphology (bar, 1 mm (i)) and trichomes (bar, 0.1 mm (ii)). (a) *Nostoc-Pc* isolated from the lichen *Peltigera canina*. This motile strain soon spreads over the culture plate and forms a mat-like colony (i). Different stages in the *Nostoc* life-cycle (ii); hormogonia (H), mature coiled filament (M). Motile hormogonia are responsible for the rapid spread of this strain. (b) *Nostoc-Pa*1 isolated from the lichen *Peltigera aphthosa*. This slow-growing strain forms pearl-like colonies (i) and has not produced hormogonia in culture. The immotile filaments are organized in dense packets (ii). (c,d) One of the three *Peltigera aphthosa* populations used in this study. The lichens are shown before (c) and after (d) removal of original cephalodia. The cephalodia (dark spots in (c)) were removed using a sterile scalpel. The scars from removed cephalodia appear as white (young thallus sections) or dark (older thallus sections) spots in (d). After the removal of cephalodia, each thallus was inoculated with a cultured *Nostoc*-strain. The lichen thalli were labelled with coloured pins, with each colour corresponding to a specific *Nostoc* strain. Five strains originated from lichens (*Nostoc-Pc* (yellow), *Nostoc-Pm* (red), *Nostoc-Nr* (orange), *Nostoc-Pa*1 (green), *Nostoc-Pa*2 (blue)) whilst two represented widely used laboratory strains (*Nostoc punctiforme* PCC 73102 (white) and *Anabaena (Nostoc)* PCC 7120 (black)). Bar, 5 cm. (e) The surface of *Peltigera aphthosa* directly after inoculation with *Nostoc punctiforme* PCC73102. The thallus surface was soaked with *Nostoc* culture, and large deposits of the inoculated strain can be seen (within grey circles). The tissue in the white rectangle is the same as that shown in (f). Bar, 1 cm. (f) After 16 wk in the field, many new cephalodia had developed on the thallus surface. Cephalodia were analysed (grey squares) both randomly and from areas where large amounts of inoculum had been deposited (compare with (e)). All analysed cephalodia contained the original *Nostoc* whilst the inoculated strain was not detected from any newly formed cephalodia. Bar, 2 mm. (g,h) Free-living cyanobacteria (within grey circles) were found growing epiphytically on several harvested lichen thalli. All these lichens had been inoculated with either strain *Nostoc-Pc* (g) or strain *Nostoc-Pm* (h). In all cases the free-living cyanobacteria represented the same strain that had been inoculated on that specific thallus. Several cephalodia were sampled (grey squares) from thallus surfaces that supported epiphytic cyanobacteria. They all contained the original symbiont: the inoculated strain was never found in a newly formed cephalodium. Bar, 2 mm.

conditions tested. However, these strains fit well into classical descriptions of *Nostoc* (Geitler, 1932; Castenholz, 1989; Komárek & Anagnostidis, 1989). The same general morphology, with trichomes occurring in packages surrounded by mucilaginous sheaths, has also previously been reported for isolates from *Peltigera aphthosa* (Whitton & Potts, 2000). It is also typical of many free-living *Nostoc* strains (Mollenhauer, 1988).

We have previously shown, using the conserved part of the trnL (UAA) intron in phylogenetic analyses, that the intron sequences of lichen-forming *Nostoc* strains group together with those of free-living *Nostoc* isolates, such as *N. punctiforme* and *N. muscorum*, but are clearly distinct from those of other Nostocalean cyanobacteria (section 4, in Rippka *et al.*, 1979), like *Anabaena azollae*, *Anabaena (Nostoc)* PCC7120, and *Scytonema* PCC 7110 (Paulsrud *et al.*, 1998; Oksanen *et al.* unpublished).

At least two of the inoculated strains, *Nostoc-Pc* and *Nostoc-Pm*, were able to live and reproduce on the surface of manipulated thalli, as they were found as large and seemingly thriving free-living colonies at the end of the experiment. Even though these cyanobacteria were abundant during the formation of new cephalodia they were never incorporated into symbiotic structures. Instead, the original cyanobiont was incorporated, even in areas of the thallus that supported free-living colonies of the inoculated strain. Figures 1g,h show some examples of free-living cyanobacteria growing close to newly formed cephalodia containing the original symbiont. Except for the two *Nostoc* strains mentioned above, none of the other foreign, inoculated strains were detected from the manipulated lichens after 16 wk in the field. Whether they were unable to survive the initial shock of inoculation or disappeared later during the experiment is not known. In particular, the two laboratory strains, *N. punctiforme* PCC73102 and *Nostoc* (*Anabaena*) PCC7120, might have lost part of their ability to survive in natural habitats during countless generations under laboratory conditions.

Of the seven *Nostoc* strains used in the inoculations, two had originally been isolated from thalli of *Peltigera aphthosa*. These two strains correspond to the two distinct *Nostoc* sequences that have been retrieved from *P. aphthosa* samples from both Finland and Sweden (Paulsrud & Lindblad, 1998; Paulsrud *et al.*, 1998). One of the strains (*Nostoc-Pa*1) shares an identical intron sequence with those found in all cephalodia analysed in this study and, thereby, presumably belongs to the same *Nostoc* strain. There is no way of finding out whether cultivated cyanobacteria of this strain were incorporated into some new cephalodia on the three *P. aphthosa* thalli that were inoculated with this particular strain. The strain was included in our experiment mainly in case many manipulated thalli either died or became unable to develop new cephalodia. In such a case it would have been interesting to investigate whether synthesis of new cephalodia would have been possible in the presence of a laboratory culture of the original strain.

The other *Nostoc* strain (*Nostoc-Pa*2) that had originally been isolated from *P. aphthosa* was not recovered from any harvested cephalodia nor was it found in a free-living form. The failure of this strain could have been caused by its short survival in the field or, more likely, because new cephalodia developed only from original *Nostoc* colonies that escaped detection during the manipulation of experimental thalli. However, the failure of this strain, along with the more general observation that there is a certain degree of cyanobacterial diversity in *P. aphthosa*, could also reflect the still insufficiently understood taxonomy of *P. aphthosa*. Five tripartite species are currently recognized in the *P. aphthosa*-group: *P. aphthosa*, *P. britannica*, *P. leucophlebia*, *P. nigripunctata*, and the recently described *P. chionophila* (Goward & Goffinet, 2000). As the taxonomy of the *P. aphthosa*-group mainly relies on a rather continuous variation of morphological characteristics and on secondary chemistry, the recognition of a morphologically and chemically variable *P. aphthosa* has been justified (Holtan-Hartwig, 1993; Vitikainen, 1994; Goward *et al.*, 1995). However, many anomalous populations exist and some of them could represent genetically differentiated entities that might also show differences in cyanobiont specificity.

The fact that only the original *Nostoc* strain was incorporated into new cephalodia raises the difficult question as to the origin of this strain. It is possible that small fragments of original cephalodia remained attached to some experimental thalli despite our careful attempts to remove all cephalodia. It is also possible that some cephalodia could have developed from primordia that escaped detection in the field due to their minute size. The third possibility is that new varieties of the correct *Nostoc* strain, originating from some source other than the original symbiotic consortium, were incorporated into the manipulated thalli.

Presuming that cyanobacteria either from remnants of old cephalodia or from sources outside the lichen were incorporated into some new cephalodia, these could have reached the site for cephalodia formation either as free-living hormogonia or in a lichenized state, as symbiotic diaspores. The latter option is supported by the results of Stöcker-Wörgötter & Türk (1994) who successfully resynthesized *Peltigera leucophlebia*, another tripartite member of the *P. aphthosa*-group. These authors found that when green, bipartite lobes of *P. leucophlebia* were inoculated with both free-living and lichenized cultures of *Nostoc*, only inoculations with lichenized cyanobacteria gave rise to new cephalodia. If this turns out to be a more general pattern among tripartite lichens, the birth of new cephalodia could only rarely reflect *de novo* lichenization, and more commonly represent the result of mechanical fusions between thallus fragments containing compatible mycobionts. However, the morphogenesis of cephalodia has been described by several authors (Forssel, 1883; James & Henssen, 1976; Ott, 1988; Rai, 1990; Stöcker-Wörgötter & Türk, 1994; Yoshimura *et al.*, 1994), and most of these descriptions indicate that cephalodia can develop via the capture of free-living *Nostoc* cells from the thallus surface.

In lichenology, where laboratory work is often slow and tedious, or even impossible, it is of great advantage if one can test hypotheses in the field before attempting laboratory studies. Lichens in general, and cyanolichens in particular, are difficult to grow under controlled conditions, and few successful attempts have been made to resynthesize cyanolichens in the laboratory (Yoshimura *et al.*, 1993; Stöcker-Wörgötter & Türk, 1994; Yoshimura *et al.*, 1994; Stöcker-Wörgötter, 1995). Stöcker-Wörgötter (1995) managed to resynthesize *P. aphthosa* from a coculture of its bionts, but it took the resynthesized thalli over a year to reach 1 cm in diameter.

This study, together with our previous studies, clearly indicates that associations between cyanobacteria and lichen-forming fungi can be very specific and stable. This is in contrast to the general view of cyanobacterial symbioses as being rather unspecific. This knowledge will be valuable when the study of these lichens is continued in the controlled laboratory environment.

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