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The *Nostoc* Symbiont of Lichens

Diversity, Specificity and Cellular Modifications

BY

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ABSTRACT

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Cyanobacteria belonging to the genus *Nostoc* have the capacity to form symbiotic associations with a wide range of organisms. Diversity, specificity and cellular modifications of the symbiosis between *Nostoc* and fungi in the formation of lichens were investigated in this thesis.

The use of the $tRNA^{Leu}_{UAA}$ intron as a genetic marker for the subgeneric identification of *Nostoc* in complex field material was developed. Lichens belonging to the genera *Peltigera* and *Nephroma* show limited variability in their *Nostoc* symbionts. The *in situ* symbiont consists of a single strain rather than a community of different *Nostoc*s, and single thalli consistently contained the same symbiont. Patterns in symbiont identity were found in geographically remote populations and the lichen species, rather than growth locality, was shown to be important for the identity of the *Nostoc* symbiont. Examination of a *P. aphthosa* photosymbiodeme revealed that one *Nostoc* has the capacity to perform the physiological roles found in both bipartite and tripartite lichens. The symbiotic association between bryophytes and *Nostoc* on the other hand exhibited a much greater variation of *Nostoc* symbionts.

Evolutionary patterns in the $tRNA^{Leu}_{UAA}$ intron were analyzed and it was shown that sequence variation was caused by several processes other than random mutations. Such evolutionary processes in genetic markers are crucial to consider, especially if phylogenetic reconstructions are attempted.

Protein profiles of symbiotic and free-living *Nostoc* were analyzed using 2-dimensional gel electrophoresis. One of the major proteins in the extracts from freshly isolated symbionts was partially sequenced and shown to contain a fasciclin domain. The corresponding ORF in *N. punctiforme* was homologous to symbiotically induced genes found in different symbiotic systems.

This thesis gives new perspectives on lichens and provides a platform for further examiniations using tools provided by modern biology.

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This thesis is based on the following papers, which will be referred to in the text by their respective Roman numerals.

I **Paulsrud, P. & Lindblad, P. 1998.** Sequence variation of the tRNA^{Leu} (UAA) intron as a marker for genetic diversity and specificity of symbiotic cyanobacteria in some lichens. *Applied and Environmental Microbiology* **64**: 310–315.

II **Paulsrud, P., Rikkinen, J. & Lindblad, P. 1998.** Cyanobiont specificity in some *Nostoc*-containing lichens and in a *Peltigera aphthosa* photosymbiodeme. *New Phytologist* **139**: 517–524.

III **Paulsrud, P., Rikkinen, J. & Lindblad, P. 2000.** Spatial patterns of photobiont diversity in some *Nostoc*-containing lichens. *New Phytologist* **146**: 291–299.

IV **Costa, J-L., Paulsrud, P., Rikkinen, J. & Lindblad, P. 2001.** Genetic diversity of *Nostoc* symbionts endophytically associated with two bryophyte species. *Applied and Environmental Microbiology* **67**: 4393–4396.

V **Paulsrud, P., Rikkinen, J. & Lindblad, P. 2001.** Field investigations on cyanobacterial specificity in *Peltigera aphthosa*. *New Phytologist* **152**: in press.

VI **Costa, J-L.*, Paulsrud, P.* & Lindblad, P.** The cyanobacterial tRNA^{Leu}(UAA) intron: evolutionary patterns in a genetic marker. *Manuscript.*

VII **Paulsrud, P. & Lindblad, P.** One of the most abundant proteins in extracts from *Nostoc* symbionts of lichens is a homologue to symbiotically induced genes from other symbiotic systems. *Manuscript.*

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PREFACE

Symbiosis as a way of living is a fascinating phenomenon. The living together of different organisms, DeBary's original definition of the word (10), includes a vast range of interesting interactions between members of virtually all organism groups.

Symbiosis also provides a powerful tool for evolution in the creation of new life forms; the eukaryotic cell is the result of a series of symbiotic events where several important components have an endosymbiotic origin such as mitochondria and chloroplasts and perhaps even the microtubular organelles (93). These symbiotic events occurred a long time ago and have developed beyond the level of symbiosis; but still today, there are different symbiotic associations with profound importance for our ecosystems such as mychorriza, the symbiosis between fungi and plants found in about 80% of all plants (130). There are also many examples of less famous and less studied symbiotic associations and others are still awaiting discovery.

The photosynthesizing and N_2-fixing cyanobacterial genus *Nostoc* participates in a wide range of symbiotic associations with hosts from different organism groups. It is clear that the other partner gains either photosynthate or combined nitrogen from associating with *Nostoc* (depending on the type of host) but the consequences for *Nostoc* to participate in these associations are not as obvious. I have in this study concentrated on the symbiotic association *Nostoc* forms with fungi – lichens. Linnaeus referred to lichens as "rustici pauperrimi" – the poor trash of vegetation (in 7). The view of lichens as something less valuable, with diffuse characteristics somewhere between plants and fungi and with limited economic importance, has influenced their study ever since, and they have not received their due attention.

The associations between *Nostoc* and fungi exhibit a rather wide range of structural and functional types (70). I believe that the different types of symbioses found in different lichens may represent different stages in a lichenisation process that could help in understanding the evolution of lichens and the mechanisms behind the wide ability of *Nostoc* to function in symbiotic associations in general.

This thesis is a small step towards such an understanding.

INTRODUCTION

CYANOBACTERIA

Cyanobacteria is a diverse group of gram-negative photoautotrophic prokaryotic organisms, all containing chlorophyll a and two photosystems (PSII and PSI) (25).

Evolutionarily, cyanobacteria are old with a fossil record possibly dating back to 3,500 million years ago (148). They include many morphological, ecological and physiological types and are found in many different habitats (170). In the nutrient poor open sea, N_2-fixing cyanobacteria such as *Trichodesmium* may form massive blooms, and unicellular genera such as *Synechococcus*, *Synechocystis* and *Prochlorococcus* often dominate subsurface waters (120). In fresh water habitats, bloom forming heterocystous forms (e.g. *Anabaena*, *Nodularia*, *Spirulina*), some of which are potentially toxin-producing, are common (143), but also unicellular types such as *Microcystis* and different Picocyanobacteria are abundant (153). On land, a large number of different genera are present in different biotopes (170). Cyanobacteria are also found in many extreme habitats, where they may dominate, including hyperthermal habitats (e.g. *Synechococcus* spp.) (166), hypersaline environments (e.g. *Microcoleus* and *Aphanotece*) (117) and deserts, both hot and cold (e.g., *Chroococcidiopsis*) (175). They are also common partners in symbiotic interactions with different organisms (e.g. *Nostoc* and *Richelia*) (1). In addition to these examples, descendants of cyanobacteria can be found as chloroplasts in plants and algae since these, according to the endosymbiotic theory, originate from an old symbiosis between eukaryotic cells and cyanobacteria (92). Chloroplasts are still genetically and structurally similar to cyanobacteria, but have lost most of their genetic material to the nucleus of the host (32).

The ecological success of cyanobacteria can to some extent be explained by their metabolic capabilities. Fundamental is the ability to perform oxygenic photosynthesis with water as a source of electrons subsequently used in the reduction of carbon dioxide just as in plants and algae (25). This enables cyanobacteria to live photoautotrophically in habitats where light and water are available. Many cyanobacteria also have the capacity to fix at-

mospheric nitrogen (170). This capability is only found among prokaryotes and has a scattered distribution among bacteria found in several unrelated groups (181). The enzyme complex responsible for nitrogen fixation is extremely oxygen sensitive, which makes it difficult to combine with an oxygen evolving photosynthesis (56). Cyanobacteria capable of both these processes have evolved different strategies involving spatial or temporal isolation of the two incompatible processes. Temporal separation may involve photosynthesis during daytime and N_2-fixation during the night (as in the unicellular cyanobacterium *Gleothece* [106]). Spatial separation involves localization of N_2-fixation to a subset of cells of the filament (as in filamentous cyanobacteria such as *Trichodesmium* [42]). In some cyanobacteria, N_2-fixation takes place in structurally and physiologically differentiated cells where oxygen tension is kept on a low level (as in *Nostoc* [2]). These cells, termed heterocysts, are protected from oxygen produced in the neighboring vegetative cells where oxygenic photosynthesis is performed, through several modifications including the following: 1) Lack of active PSII (and thus no O_2 evolution), 2) additional cell wall layers (one glycolipid- and one polysacharide layer), and 3) high rate of respiration (39). In these filaments an exchange of metabolites takes place with vegetative cells providing photosynthate in exchange for fixed nitrogen from the heterocysts (172). The regulation of spacing and development of heterocysts is still not fully understood, but many key factors have been analyzed in recent years (2, 178). It has been suggested that some 15–25%, or 600–1,000 genes, of the genome of *Anabaena variabilis* are heterocyst-specific (90). Genome comparisons between heterocystous and non-heterocystous strains will provide further information on this.

So far, there is only one completed cyanobacterial genome publicly available: the unicellular non N_2-fixing strain *Synechocystis* PCC 6803. This was the first genome from a photosynthetic organism completed and, with its 3.57 Mb, the largest when it became available in 1986 (77, 78). Since then, a large number of cyanobacterial genomes have been initiated, and draft versions are available for several strains (such as the heterocystous filamentous strains *Anabaena* PCC 7120 – 6.37 Mb [http://www.kazusa.or.jp/cyano/anabaena/] and *Nostoc punctiforme* ATCC 29133 – 7.5 Mb [http://www.jgi.doe.gov/JGI_microbial/html/*Nostoc*/*Nostoc*_homepage.html]). Comparative analyses of these genomes may solve many problems in cyanobacterial biology, but also introduce new questions.

SYSTEMATIC AND TAXONOMIC SITUATION

The systematic and taxonomic situation of cyanobacteria is somewhat unclear. Until the end of the 20th century, cyanobacteria (blue green algae) were treated together with algae, and thus fell under the botanical code of classification. When the true prokaryotic nature of the cyanobacterial cell was revealed, the need for a new classification for cyanobacteria became evident. The different perspectives of the botanical and microbiological taxonomical codes make them suitable for different fields of cyanobacteriology. For example, when type specimen under the bacteriological code should be an axenic culture deposited in a culture collection, the type specimen under the botanical code should be a dried field sample deposited in a herbarium. From the botanical perspective, the bacteriological approach is unsatisfactory as many of the interesting features seen in the field are lost when the cyanobacterium is cultured and there is risk that cultures mutate and thus change with time in a culture collection. From the bacteriological perspective, the botanical approach is unsatisfactory as field samples often contain many different genotypes of more or less closly related strains and living material is often needed in order to perform tests important in prokaryotic taxonomy (23). Problems to combine the different systems have resulted in the parallel use of different taxonomic schemes often containing the same genus names, but with somewhat different descriptions. Scientists dealing with cyanobacteria in the field (limnologists, ecologists, etc.) generally use a botanical system (46, 85), whereas those working with strains from culture collections (physiologists, molecular biologists) use the emerging bacteriological system (142). In the second edition of *Bergey's Manual of Systematic Bacteriology* (25), a phylogenetically based system is still not implemented. Instead, the suggested system is based on a revised version of the previous bacteriological systems (25, 142), which in turn are simplifications of previous botanical systems, but where only characters readily observable in culture are considered. The cyanobacterial phylum is divided into five subsections (25):

I - Unicellular strains reproducing by binary fission.
II - Unicellular strains reproducing, at least sometimes, by multiple fission.
III - Filamentous strains with division in one plane and without heterocysts.
IV - Filamentous strains with division in one plane and with heterocysts.
V - Filamentous strains with division in more than one plane.

This system is unsatisfactory from a phylogenetic point of view, but is kept until the "true phylogeny" of cyanobacteria can be resolved (25).

Delimitation of species in cyanobacteria can not yet be performed under the bacteriological code (25), but in the botanical literature there is a large number of species and species descriptions currently in use (17, 46, 85). A phylogenetically based molecular system may be able to incorporate aspects and names from previous systems in a way that is acceptable for all cyanobacteriologists. The large scale consistency between botanical, bacteriological, and molecular groupings (at least for the more complex filamentous types) gives some hope for this (24, 100). Species names in prokaryotes will always be arbitrary, but it is often operational to have names for different adaptive peaks in the continuum of bacterial diversity (19, 24).

NOSTOC

The bacteriological definition of *Nostoc* is in summary:
Nostoc is a cyanobacterial genus characterized by a complex life cycle. Trichomes are untapered and consist of cylindrical or spherical cells. Hormogonina, the motile stage in the *Nostoc* life cycle, give rise to young filaments with terminal heterocysts at both ends. Intercalary heterocysts are formed in mature filaments. Akinetes (spores), often in chains, are formed distant from heterocysts (25). Most definitions emphasize the complex life cycle, but different weight is placed on such characters as the gelatinous colony stage and characteristics of akinetes, heterocysts and hormogonia (17, 46, 85, 142).

Often occuring as macroscopic colonies, *Nostoc* has been described and used since long before bacteria in general were discovered. In China, colonies of *Nostoc commune* were thought to have extraordinary abilities and were used as food, perhaps as early as 300 AD. In Europe, the first clear record of *Nostoc,* as well as the name, probably comes from the 16th century philosopher Paracelsus (128, 129). However, the first definition of *Nostoc* comes from J-P. É. Vaucher (1763–1841) and since then, numerous botanists and microbiologists have worked with this genus (102).

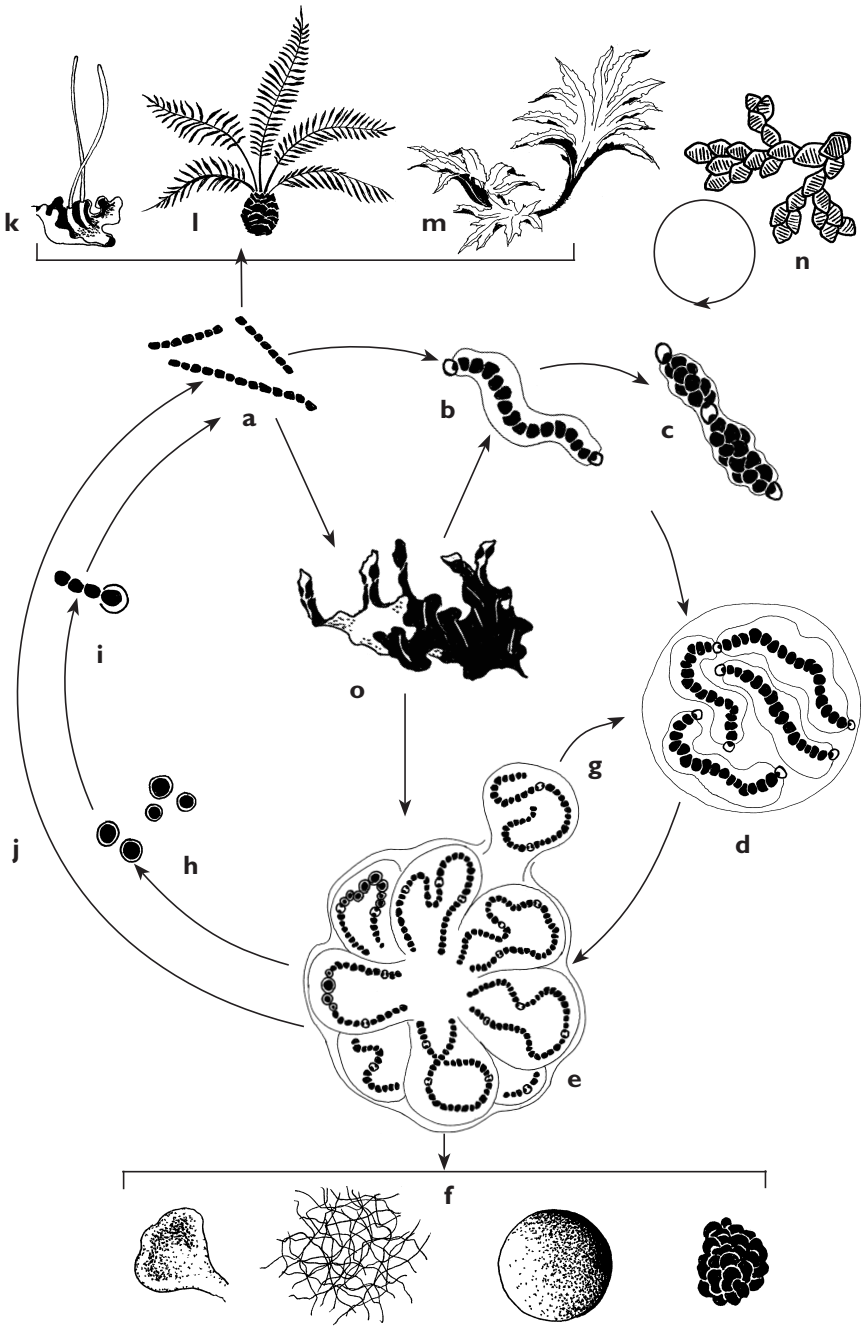
Nostoc is an ecologically successful genus which can be found in a large number of biotopes from the tropics to the arctic. It is a common inhabitant of humid soils, fresh water habitats, and seasonally inundated habitats but almost absent from marine habitats (33).

Most accounts of free-living *Nostoc* are based on macroscopically visible colonies, and the distribution and abundance of *Nostoc* as microscopic colonies or single filaments in terrestrial habitats is largely unknown (33, 100).

FIG. 1. The *Nostoc* life cycle (modified and extended from 100) contains different cell types and developmental alternatives. Some species may only be capable of part of this life-cycle (100).

Hormogonia (**a**) are the motile filaments in the *Nostoc* life cycle and serve as dispersal units that can migrate to places where new colonies can be established. Hormogonia are also the infective unit in most symbiotic associations. The mechanisms behind the gliding motility of these cells are only partly understood (63). Hormogonia are induced by a range of factors (e.g. red light, solid substratum, new medium, or factors from the symbiotic host [96]). The differentiation of hormogonia to vegetative filaments (**b**) involves the development of heterocysts at each end (at least under nitrogen deprived conditions), enlargement of the cells and formation of a sheath around the filament. Further development of the vegetative filament results in longer filaments, in some cases extremely contorted and wrinkled, possibly as a consequence of a hardened sheath (**c**) (100). Intercalary heterocysts are now formed. Continuous growth and fragmentation of filaments leads to the formation of a larger colony (**d**, **e**), sometimes reaching macroscopical dimensions (**f**). Proliferation (**g**) (100) may occcur through budding of the mature colony. In response to harsch conditions (e.g. phosphate or energy limitation), some strains may develop akinetes (**h**). These start forming distant from heterocysts, and often occur in rows. Germinating akinetes, or resting spores (**i**), give rise to new hormogonia. New hormogonia may also form directly from vegetative filaments (**j**).

Different hosts (**k–o**) forming endophytic symbiotic associations with *Nostoc* . In at least the bryophyte (**k**), cycad (**l**) and *Gunnera* (**m**), it is the hormogonium which is the infective unit. In *Azolla* (**n**), the symbiont is transferred from generation to generation, and infection of new leaf cavities by hormogonia-like filaments occurs from an apical colony as the plant grows (125). In the case of lichens (**o**), the importance of the hormogonial stage is less clear. Most lichens grow out from an undifferentiated pre-thallus. Whether this is formed from hormogonia or develop from vegetative cocultures of mycobiont and *Nostoc* is not known. In the case of new cephalodia, an important role for the hormogonium has been suggested also in lichens (151). In the lichen genus *Collema* (see Fig. 3), colonisation of mature colonies have been proposed (31), and in the association between *Nostoc* and the Zygomycete *Geosiphon puriforme*, the primordial stage (**b**) is where the fungus can incorporate *Nostoc* (83).



N. commune *N. commune* var. *flagellifor-* *N. pruniforme* *N. zetterstedtii*

The gelatinous colonies of *Nostoc,* where a large number of filaments reside, are important in the capacity to withstand desiccation as well as UV irradiation (**127**). The extracellular carbohydrates have a great water storing capacity and, in addition, frequently contain UV absorbing pigments such as scytonemin (**129**). The different colony morphologies are often lost when strains are brought into culture (**142**) and colony morphology may reflect growth conditions rather than genotype. This can be demonstrated by *Nostoc commune* var. *flagelliforme* forming hair-like threads in the field, but, in culture, forming *Nostoc commune* colonies (**100**). This plasticity may be related to the complex developmental repertoire of *Nostoc*. The life cycle of this genus is schematically represented in figure **1**. A single *Nostoc* strain may not be capable of all these developmental alternatives, and different varieties could be presented for the different stages. Included are also some common macroscopical colony types and some hosts with which *Nostoc* forms stable endophytic symbioses.

Nostoc is also rather flexible physiologically and metabolically. In addition to autotrophic growth, many strains grow well chemo-organotrophically in the dark with a range of carbon sources such as sucrose, fructose, and glucose. Some strains may also ferment sugars in anoxic conditions (**150**). Nitrogen is preferrably taken up as ammoniun, but nitrate and urea as well as dinitrogen (in nitrogen fixation) can also be utilized (**59**). Most studies on the physiology and metabolism of *Nostoc* have been performed in exponential phase cultures. These cultures have a doubling time of about **24** hours and grow in a medium containing a rich supply of macro- and micro-nutrients. The natural conditions are quite different. Growth is slow and there is often competition for the small amounts of nutrients available. In fact, many *Nostoc* seem to be threatened by eutrophication in their natural habitats (**101**), and the distribution of many species is restricted to oligotrophic waters (**100**).

An increased knowledge of physiological responses to many different conditions and stationary phase, as well as the different stages in the *Nostoc* life cycle, are thus required to understand the full potential of *Nostoc*.

SYMBIOTIC ASSOCIATIONS WITH NOSTOC

Nostoc forms symbiotic associations with a large number of organisms from different taxonomic groups (see Fig. **1**, table **1**). Among plants, endophytic symbiotic associations with representatives from bryophytes (mosses, hornworts and liverworts), pteridophytes* (the water fern *Azolla*), gymnosperms (cycads), and angiosperms (*Gunnera*) (**1, 96, 133**) are formed. The association with fungi in the formation of lichens will be discussed in the next section. In all these plants, the general structure in which *Nostoc* is housed is also formed in the absence of a symbiont (**73, 125, 133, 168**). The symbiotic plant structure is then colonized by hormogonia which may be induced by and attracted to the plant host (**21, 28, 51, 84, 135**). In the case of *Azolla*, the *Nostoc* symbiont is not obtained from the surrounding, but is transferred over the

Host	main group	subgroup	Position of *Nostoc*	I/E	Het. freq.[1)]	Transfer from: *Nostoc*	Host	Ref.
fungus	ascomycete	bipartite sp.	thallus	E	**2–7%**	glucose, NH_4^+	-	**132**
		tripartite sp.	cephalodium	E	-55%	NH_4^+	-?	**62, 132**
	zygomycete	*Geosiphon*	bladder	I	3–10%[2)]	PS, N	-	**83,103**
bryophyte	liverwort	few	gam. cavity	E	-35%	NH_4^+	PS	**95,144**
	hornwort	most	gam. cavity	E	-45%	NH_4^+	PS	**95**
	moss	*Sphagnum*	hyaline cells	E	?	N		**95**
pteridophyte		*Azolla*	leaf cavity	E	-30%	NH_4^+	PS	**125**
gymnosperm	cycad	all	coralloid root	E	-45%	gln or cit/gln	PS	**88, 123**
angiosperm		*Gunnera*	stem gland	I	-60%	NH_4^+	PS	**12**

Table **1**. The different groups of organisms which form endophytic associations with *Nostoc* and some of the characteristic features of these associations. References are mostly reviews. I/E – intracellular or extracellular.
gam. – gametophyte, NH_4^+ – ammonium, N – unknown nitrogen species, PS – photosynthate, cit – citrullin, gln – glutamine.
1) Heterocyst frequencies. There is often a gradient with heterocyst frequencies similar to free-living strains in newly colonised structures, and increasing frequencies in older tissue. Upper limit frequencies shown in this column.
2) Heterocyst frequency reported as unaltered from free-living state i.e. about 3-10 %.

* The symbionts of *Azolla* are often referred to as *Anabaena*, but several studies indicate that they are closer to *Nostoc* (**125, 126**).

generations via the sporocarp. Hormogonia-like filaments from a plant associated colony are however responsible for the infection of each new cavity (**125**). These hormogonia subsequently develop into mature filaments with differentiated heterocysts. In these associations it is the capability of *Nostoc* to fix atmospheric nitrogen which is interesting for the photosynthesising host. In general, in plant symbioses, the *Nostoc* symbiont is believed to obtain a carbon source from the host in exchange for the product of N_2-fixation, and heterocyst frequencies in the symbiotic condition are highly elevated as compared to the free-living state (see table **1**).

Several lines of evidence, including high concentrations of ammonia (normally repressing heterocyst formation) in the symbiotic cavity and presence of cyanophycin granules (storage compound for nitrogen) in symbiotic cells, indicate that not nitrogen starvation, but a different signalling pathway for heterocyst formation is active in symbiotic systems (**1, 96**). It is not only the high frequency of heterocysts that is special for the symbiotic mode of life, cells are larger, and a high metabolic rate can be maintained despite minimal growth once *Nostoc* has filled the symbiotic space.

From studies on axenically grown plants it has been shown that many different *Nostoc* strains from different sources can infect a single plant species. These experiments have been performed on several plants such as the hornwort *Anthoceros punctatus* where both free-living and symbiotic strains isolated from lichens, cycads, *Gunnera* and bryophytes were used to reconstitute the symbiosis (**35**), *G. manicata*, which also could be infected with strains isolated from all these symbioses (**74**), and the cycad *Zamia furfuracea*, which could be infected with both its original symbiont and a strain isolated from the soil (**119**). The diversity of *Nostoc* symbionts in natural habitats is less known, but different methods resulting in banding patterns (fingerprinting, DGGE, and RFLP) on the symbionts of *Azolla* (**82, 161, 184**), *Gunnera* (**115, 137, 138, 185**), the hornwort *Phaeoceros* (**169**) and cycads (**89**) as well as sequencing based methods on symbionts of cycads (**29**) and *Azolla* (**114**) have been performed. The studies are difficult to compare as the methods have different resolution and different types of material were used (isolated strains, field material, green house material). However, diversity rather than specificity is a general pattern in all cases except for *Azolla* where the phylogeny of symbionts parallelled that of the host (**114**).

Nostoc strains cultured from different symbiotic associations have been used in a large number of studies. Confirming the identity of the strain before and after isolation would, however, be a desirable procedure. This has

rarely been done with strains of symbiotic origin in use today. The strain used as a reference strain for the association with *Azolla*, *Anabaena azollae*, proved after closer examination not to be the main but a minor symbiont (45, 125). There is still no report on successful isolation of the main symbiont from this association. This could well be explained if the symbiont has lost its ability to grow free-living as a consequence of the fact that it is following its host over the generations and thus never needs to go through a truly free-living phase. The culturing of the "wrong" strain should not be as problematic for the other plant symbioses where each generation might require new colonization.

LICHENS

Lichens are symbiotic associations between a mycobiont, fungus, and a photobiont, alga and/or cyanobacterium. Estimates of the number of lichen species range from 13,000 to 17,000, of which about 10–15% contain a cyanobacterial symbiont (61, 108, 140). The most frequent cyanobacterial genus in lichens is *Nostoc*, but genera such as *Scytonema* and *Calothrix* are also common (43, 156). Lichen systematics and taxonomy are based solely on the fungal partner (4) but the importance of the photobiont in lichen systematics has been debated (4, 134). Even though a large majority of lichens are ascomycetes, lichenized representatives with a cyanobacterial photobiont are found in separate fungal lineages (ascomycetes, basidiomycetes and zygomycetes*) and so a polyphyletic origin of this life form is assumed (44).

Most lichens are slow-growing organisms and many grow where other organisms fail – in deserts, arctic environments, and on exposed surfaces. The physiology of lichens is generally characterized by extreme resistance (to drought, cold, heat, UV-radiation) and at the same time sensitivity (to pollution, fertilization). However, many different ecological strategies are found within the diverse lichen group (81, 108).

Lichens are poikilohydric organisms whose water status varies with surrounding conditions. Many lichens go through almost daily cycles of hydration and drying where photosynthesis is rapidly resumed after rehydration. They are known to tolerate extreme temperatures when desicated (short exposure to above 90°C and several days exposure to -196°C have been

* The only case where the host is a zygomycete is the species *Geosiphon puriforme*. This symbiosis, in which the *Nostoc* is captured intracellularily in a bladder (104) is traditionally not treated together with lichens, but following the definition above is included in this group.

demonstrated) (109). It is believed that the high levels of polyols and polyamines protect protein conformation during desiccation so that growth can be resumed quickly after rehydration (66, 108).

Figure 2 summarizes the life cycle of a lichen (of the general type used in this thesis) with emphasis on the association with a suitable photobiont. Several structures of potential importance for their reproduction can be described, but the relative importance of different strategies is harder to elucidate (70). Many lichens contain both sexual and asexual modes of reproduction. Reconstitution experiments using different cyanobacterial strains, similar to those described for plants, have not been performed except for the association between the zygomycete *Geosiphon* and *Nostoc*. A large number of *Nostoc* strains, free-living as well as from different other symbioses, were able to form this intracellular symbiosis (103).
There is great diversity in the structural organization and complexity of different lichens (57), also for *Nostoc*-containing lichens (see Fig. 3). The thallus in the lichen genus *Collema* resembles a cyanobacterial colony penetrated by fungal hyphae (FIG. 3a). The fungus seems to have limited effect on the morphology and organisation of the *Nostoc* filaments, but is important for the lobation of the thallus (31). In the lichen genus *Peltigera* on the other hand, the thallus is quite complex, and *Nostoc* is altered in morphology and confined to a special zone of the thallus (15, 163). In bipartite *Peltigera* where *Nostoc* is the only photobiont, it is found evenly distributed in a zone below the upper cortex of the thallus (FIG. 3b, FIG. 4). An even more complex lichen is *P. aphthosa*. This is a tripartite lichen where the thallus contains two different photobionts in addition to the fungal host. The primary photobiont is the green alga *Coccomyxa* which is evenly distributed under the upper cortex much like the cyanobacterium in a bipartite *Peltigera*. The *Nostoc* cells on the other hand are confined to specialized structures: cephalodia (FIG. 3c) (163). The cephalodia are situated on the upper cortex of the thallus, but may in other tripartite species be internal (as in *Nephroma arcticum*) or situated on the lower surface (as in the more loosely associated cephalodia of *P. venosa*).

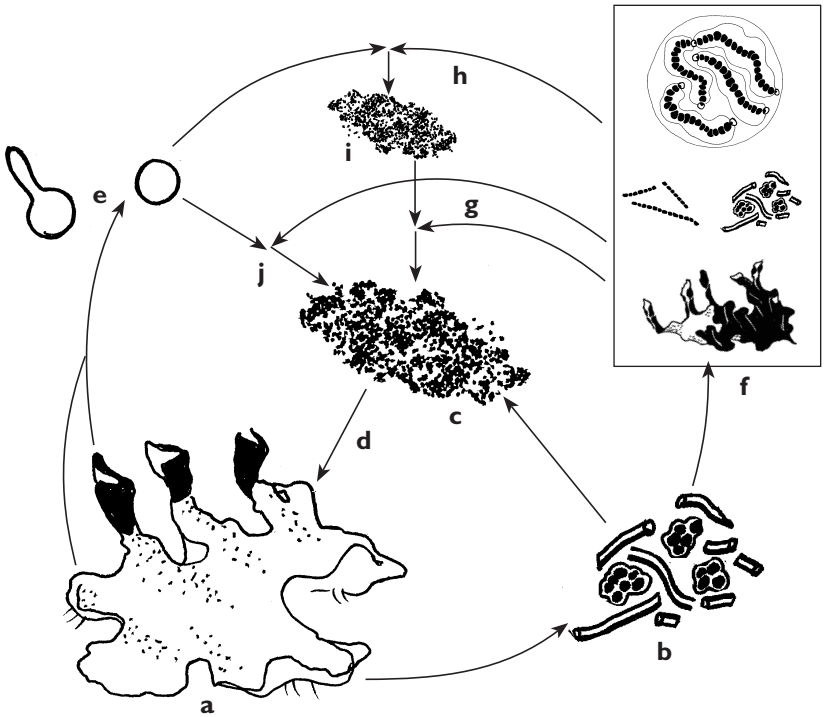


FIG. 2. Schematic representation of different possible reproductive strategies in lichens with an emphasis on the symbiotic nature of lichens (II, 68).

The mature thallus (**a**) may contain structures specialized for the dispersal of the mycobiont alone as well as together with its photobiont. When the fungus is dispersed as propagules of photobiont and mycobiont together (**b**) (e.g. soral, isidia , thallus fragment) these can readily establish a new lichen. First, an undifferentiated prethallus (**c**) will be formed followed by the formation of the more organised and stratified thallus (**d**). Both asexual (conidia) and sexual spores (ascospores) exist for the spread of the fungus alone (**e**). These spores need to associate with a photobiont. This photobiont may be obtained from different sources (**f**) such as another lichen or lichen propagule (of the same or a different species) or free-living photobionts (hormogonia or mature colony). In all these cases, the obtained photobiont may be compatible (**g**) or not (**h**). If the photobiont is not compatible, the association will never proceed beyond the prethallus stage (**i**). This stage may however provide a way of survival until a compatible photobiont can be obtained (**g**) (from any of the sources mentioned above). The fungal spore may also associate with a compatible photobiont from the beginning (**j**). After associating with a compatible photobiont, development can proceed to a mature thallus (**d**). The type of signalling required to start the differentiation into a mature stratified thallus is unknown.

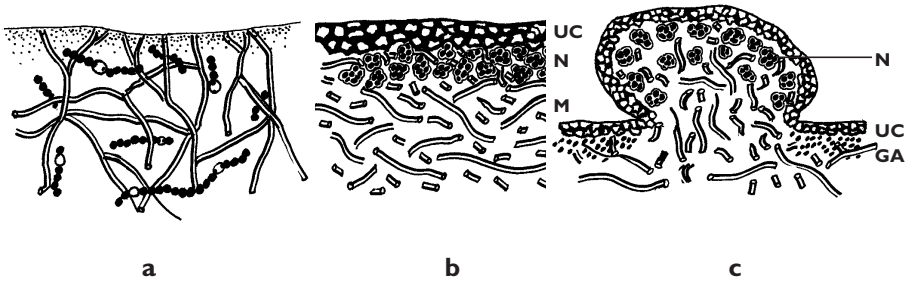


FIG. 3. Schematic representation of different thallus types of *Nostoc* containing lichens. (**a**) Example of a less organised lichen, such as in the genus *Collema*, where the structure resembles a cyanobacterial colony penetrated by fungal hyphae. (**b**) In more complex lichens, such as bipartite *Peltigera* species, the mycobiont is responsible for the structure of the thallus with an upper cortex (**UC**), photobiont layer to which the *Nostoc* (**N**) cells are restricted, and a medulla (**M**). (**c**) In tripartite lichens, such as *P. aphthosa*, a green alga (**GA**) is located in a layer underneath the upper cortex much like the *Nostoc* cells in the bipartite *Peltigera*. In this type, the *Nostoc* cells are confined to specialized structures, cephalodia, located on the upper surface of the lichen.

The *Nostoc* symbiont of lichens provide both photosynthate and fixed nitrogen to their symbiotic partners. The relative importance of these two functions may vary between different types of lichen symbioses. In bipartite cyanolichens, cyanobacteria are the only photosynthetic component having a major role in photosynthesis. Conversely, in tripartite lichens, the green alga produces most of the photosynthate. In this situation the *Nostoc* symbionts, restricted to cephalodia, mainly function in N_2-fixation (132).

The *Nostoc* cyanobionts of tripartite, cephalodiate lichens generally show higher heterocyst frequencies and higher rates of nitrogen fixation than free-living cyanobacteria. Comparable increases do not generally occur in the cyanobionts of bipartite cyanolichens. The heterocyst frequencies of lichenized, cephalodial *Nostoc*s commonly range from 15 to 35% of trichome cells, as opposed to the 5 to 10% of free-living *Nostoc* trichomes and lichenized cyanobionts of bipartite cyanolichens (81, 131).

In the genus *Peltigera*, which consists of both bipartite and tripartite members, it has been suggested that the bipartite cyanobacterial type may be ancestral and that green algal containing species developed from cyanobacterial morphotypes (97). It has been shown that green algal thalli of the

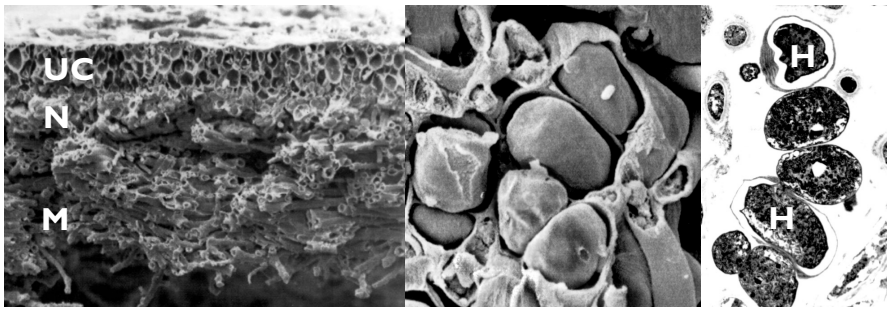


FIG. 4. Electron micrographs of the *in situ* cyanobionts of lichens belonging to the genus *Peltigera*. (**a**) Scanning electron micrograph of a cross section of a bipartite species showing the stratified thallus with an upper cortex (**UC**), *Nostoc*-layer (**N**) and medulla (**M**). (**b**) Detail from (**a**) showing a *Nostoc* "package" surrounded by fungal hyphae. (**c**) Transmission electron micrograph of *Nostoc* filament in a cephalodium of *P. aphthosa*. Two heterocysts (**H**) are seen separated by two vegetative cells.

tripartite species *P. leucophlebia* (152), *P. aphthosa* (151), *P. venosa* (118) and *P. britannica* (20) develop from cyanobacterial crusts or from small bipartite cyanobacterial thalli.

Lichens in general, and cyanolichens in particular, are difficult organisms to grow and reconstitute from their isolated bionts (30). However, the number of lichen mycobionts that have been cultured increases steadily with improved culture methods (30). The mycobionts from *Peltigera* species were considered particularily difficult to grow without the photobionts (3), but work by Stöcker et. al. (151, 152) provided a method for both isolating the mycobiont and resynthesizing the lichens from the isolated bionts for this genus. The resynthesis is performed through co-culture of the bionts on soil substrate subjected to cycles of wetting and drying (152). The development of the tripartite lichen *P. aphthosa* may serve as an example of the events leading to the formation of a mature thallus. The first thallus primordia (both cyanobacterial and green algal types) start forming on the cyanobacterial crust after 3–4 months. After 5–6 months small prothalli develop and after 8 months the first corticated small thalli are formed. After 14–16 months cephalodia can be found on the green lobes (151). The successful resynthesis of these lichens allows for a more experimental approach in their study, but the time factor makes this work very time consuming.

AIM OF THIS PROJECT

The questions addressed in this project cover two main aspects of lichen biology:
1) Distribution and specificity of *Nostoc* in relation to different hosts.
2) Interactions between the symbionts and the basis for the modifications of *Nostoc* in the symbiotic state.
The difficulty to maintain lichens in the laboratory and to resynthesize them from their isolated bionts (**30**, **151**) has made them a largely neglected group of organisms among physiologists and molecular biologists. Using some of the opportunities offered by modern biology and applying them to the lichen symbiosis can be useful for both the field of lichenology and cyanobacteriology.

A clear view of the diversity and host specificity of *Nostoc* symbionts is necessary in order to proceed with resynthesis experiments or molecular experiments where probes and primers directed against specific target sequences are used. Thus, my work has concentrated on the first of the two aspects, with only initial work on the second aspect.

The knowledge available when the project started stated that many lichens contain cyanobacteria belonging to the genus *Nostoc*. The altered morphology of the cyanobiont (**13, 15, 79**) in symbiosis, in combination with the lack of a clear taxonomy, has made any subgenus identifications of the *Nostoc* symbionts impossible (**58**, **103**).

The diversity of *Nostoc* symbionts on different levels was thus one focus in this thesis. Is there one or are there several different *Nostoc*s in
– one "point" of a thallus?
– one thallus?
– one population?

Questions on a higher level include the following:
– Are there patterns in the distribution of different *Nostoc* symbionts that are correlated to either different hosts or different geographical areas?
– Are there different *Nostoc*s involved in the physiologically different bi– and tri-partite associations?
– What is the relationship between the *Nostoc*s found in lichens and the soil community or other symbiotic systems (bryophytes) at the site?
– How do patterns of diversity compare between lichens and other symbiotic systems?

The second aspect, on which initial work has been performed, focuses on the modifications of *Nostoc* in lichens. There are both similarities and differences between the symbiotic associations *Nostoc* forms with fungi and plants. Within lichens there are differences between lichens with different degrees of organization as well as between the bi- and tri-partite lichens.
– What are the major physiological modifications of *Nostoc* in lichen symbioses?
– What are the physiological differences between the *Nostoc* symbiont(s) in bi- and tri-partite lichens?

All work was done on lichens of the genera *Peltigera* and *Nephroma* in the suborder Peltigerineaea of the order Lecanorales (**36**). Many of the cyanolichens previously used in physiological and ultrastructural work belong to these genera, and there are both bi- and tri-partite representatives.

BIOLOGICAL MATERIAL

Field material has been used consistently in this thesis. Studies on diversity and specificity of *Nostoc* symbionts were performed on intact thalli brought to the laboratory. Direct examination of these thalli, with as few steps as possible and mixed multiple sampling, is crucial in order to avoid artefacts and contamination.

The studies started on single thalli from one locality in northern Dalarna in Sweden. As a consequence of the results obtained, sampling was subsequently expanded to biological material from several localities in northern Dalarna as well as localities in Finland and in USA. In order to compare the situation in lichens with other symbiotic systems, the diversity of *Nostoc* symbionts in bryophytes was also examined. Representatives of liverworts and hornworts that form stable endophytic associations with *Nostoc* and grow in the same habitats as the investigated lichens were used. Field inoculations were performed at the same locality where specificity and diversity had previously been examined.

In the second part of this thesis, the examination of alterations in *Nostoc* in the symbiotic state, field material was used as well. Cyanobacterial symbionts were purified from freshly collected lichens and used for further analyses. This approach has the advantage that artefacts introduced by laboratory procedures are avoided, but makes it more difficult to control the history and status of the biological material and thus to repeatedly obtain comparable biological material.

GENETIC MARKER

The study of microbial ecology has gone through a revolution with the introduction of molecular markers. Identification of bacteria previously required pure cultures, and only a fraction of all bacteria in the field could be cultured (5). Molecular markers allow for the identification of different DNA species in mixed and unculturable microbiological communities. Different markers provide identification on different levels and the type of question(s) will thus influence what marker(s) will be used (186).

In cyanobacteria, diversity and phylogenetic relationships have been examined using a range of markers and techniques including nucleotide sequence of *nifH* (155, 182, 183), 16S rDNA (72, 112, 113, 147, 157, 171), *rpoC* (121, 122), *cpc*-locus (8, 9, 111), *hetR* (72), $tRNA^{Leu}_{UAA}$ intron (14, 124, 145, 146) as well as RFLP (16, 37, 91, 138), DGGE (41, 138) and finger-printing methods (110, 149, 137).

In order to answer the problems and questions outlined above, the molecular marker needs to fulfill the following criteria:

– Specific amplification from complex samples with several DNA species in order to use field material directly.

– Target gene sufficiently variable on a subgeneric level with biological variation distinguishable from potential PCR errors.

– A single copy marker (intercistronic variation may cause problems on this low taxonomic level) that is not laterally mobile.

– Small size for easy sequencing.

Three regions where sufficient variation could be expected were considered: *nifS-nifU* spacer, $tRNA^{Leu}_{UAA}$ intron, and 16S – 23S ITS. In initial experiments, all these regions were evaluated, but since the $tRNA^{Leu}_{UAA}$ intron seemed to meet these criteria, it was used in all further studies.

The $tRNA^{Leu}_{UAA}$ intron was the first intron discovered in bacteria (176). It was found in cyanobacteria, interrupting the $tRNA^{Leu}_{UAA}$ gene in the anticodon (between bases 2 and 3) and was shown to have autocatalytic activity (Fig 5). A similar intron was also found in the same position in the genome of plant chloroplasts (86). Finding closely related intron sequences in the same position of cyanobacteria and chloroplasts lead to the theory that this is an ancient and stable genetic element dating back to before the incorporation of chloroplasts (124). Loss of intron in a few cases would account for the observation that some cyanobacterial lineages lack an intron

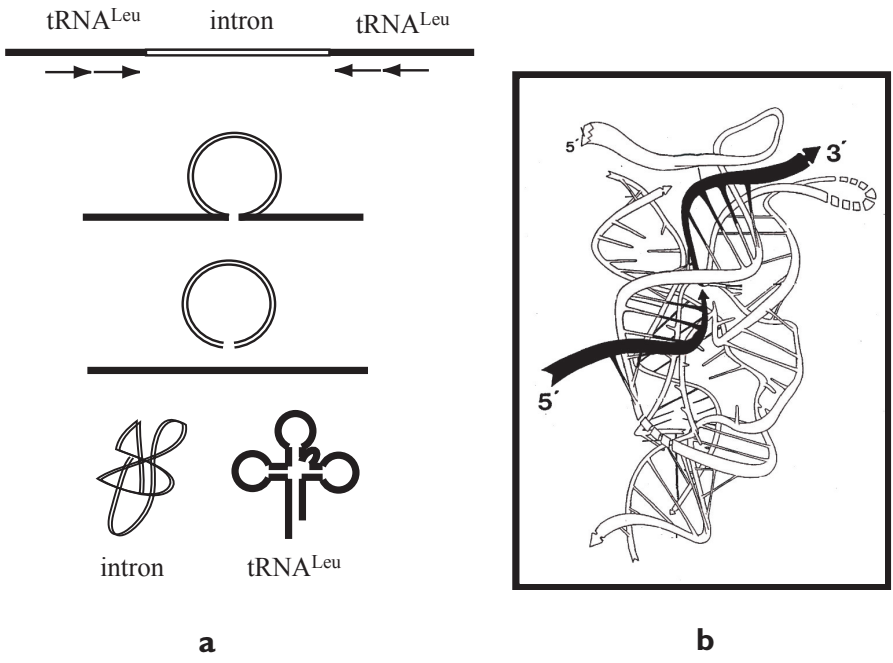


FIG. 5. The $tRNA^{Leu}_{UAA}$ intron is interupting the trnL gene in the anticodon. The intron has autocatalytic capacity and thus cleaves itself out of the transcribed gene (**a**). Regions flanking the intron are conserved and possible to use for primer construction (arrows). The structure of the intron (**b**, modified from 99) contains a highly conserved core required for the autocatalytic activity, and peripheral regions showing great variability.

in this position (124). The catalytic core (Fig 5b), vital for the autocatalytic activity, is conserved (26, 99) whereas more peripheral regions show variability on a subgeneric level (176).

RESULTS AND DISCUSSION

DIVERSITY AND SPECIFICITY OF SYMBIOTIC *NOSTOC* (PAPERS I–IV)

Methodology

A method was developed that allowed for the study of diversity of *Nostoc* symbionts on a subgeneric level. It is based on amplification of the tRNA$^{Leu}_{UAA}$ intron using degenerate primers directed against the conserved tRNA gene, without the need for DNA extraction. The sample size can be kept to a minimum making it possible to investigate many separate samples from a small sample areas. The nucleotide sequences of the resulting amplification products are analyzed in the biological context of the samples.

The choice of method and marker aimed at avoiding problems encountered when analyzing bacterial diversity in the field (**164**). In contrast to methods not based on determination of nucleotide sequence, such as fingerprinting methods and RFLP, sequence information can confirm the cyanobacterial origin of variation. Intercistronic variation is another concern, especially since closely related strains were examined. It is often believed that variation between different copies of, for example the rRNA operon, is negligible as a consequence of concerted evolution (**87**). However, studies showing variation between copies of rRNA genes exist from all organism groups (**22, 107, 165, 177**). For the cyanobacterial 16S–23S ITS sequence, variation has been shown within one strain on several occasions (**18, 69**). As the *trnL* gene is a single copy gene, this problem can be avoided.

Diversity in Single Thalli

Since the diversity of *Nostoc* symbionts in cyanolichens, as well as in other systems, was largely unknown, examination of the diversity began on a small scale.

From single samples (single cephalodia or mm pieces of lichen thalli) many clones from a single PCR amplification were analyzed for heterogeneity. The procedure was performed repeatedly for many species without heterogenity within one sample ever found (Fig. 6a). To ensure that this was not an effect of primer bias, different mixes of free-living isolates and thalli

fragments were used to successfully amplify different tRNA$^{Leu}_{UAA}$ introns from single reactions (unpublished). Furthermore, cyanobacterial strains cultivated from lichen thalli had identical sequence to that found in the intact lichen indicating that direct amplification correctly reflects the situation in the symbiosis. As mentioned earlier, attempts to culture the main symbiont of *Azolla* have failed (**45, 125**). Furthermore, Miao et. al. (**98**) investigated the *Nostoc* symbionts of the lichen *Peltigera membranacea* using the **16**S rRNA gene. Two different sequences were found in the intact thalli of different colormorphs of this lichen. However, an attempt to culture these resulted in strains containing only one of the sequences, also from the thallus where the second sequence type had been detected (**98**). Genetic differences between cultured and *in situ Nostoc* symbionts in the lichen *Nephroma laevigatum* have also previously been reported (**80**). Examining diversity of symbionts using isolated strains will always introduce a factor of uncertainty compared to working with field material.

The same homogeneity within one sample as observed for lichens was also observed when single cavities of the liverwort *Blasia* and hornwort *Anthoceros* were examined using the same technique (paper IV).

When analyzing many samples from a single lichen thallus the results were the same in all but one case. Both in bi- and tri-partite species, a single lichen thallus consistently contained only one intron type (Fig. 6b). This was found consistently for the lichens *P. aphthosa*, *P. canina*, *P. membranacea, P. neopolydactyla*, *P. britannica, N. arcticum* and *N. resupinatum*. This result indicates that the lichen, once associated with a cyanobiont, does not incorporate new cyanobacterial strains from the soil, but rather grows together with its original symbiont. In bipartite lichens, where the cyanobacterial zone is continous, this is easily envisioned, but in tripartite associations where the cyanobacterial colonies are isolated, spread of the cyanobiont over green algal sections must occur. An observation from resynthesis studies supports the spread of the already lichenized cyanobacterial strain to new cephalodia. In *P. leucophlebia*, new cephalodia only formed from *Nostoc* filaments already infected with the fungus and not from truly free-living filaments (**151**). However, the morphogenesis of cephalodia has been described by several authors (**71, 75, 118, 180**), and most of these descriptions indicate that cephalodia can develop via the capture of free-living *Nostoc* cells.

The exception from this pattern of homogeneity was the tripartite lichen *P. venosa*. On several occasions different intron sequences were amplified from different cyanobacterial colonies associated with individual *P. venosa*

thalli. From one thallus, three different intron sequences were amplified. This is the only lichen species where more than one sequence associated with a single lichen thallus has been detected. However, subsequent analyses of more thalli of this species showed that this diversity may not always be found (unpublished). *P. venosa* is a taxonomically uniform, highly distinctive species (**97, 163**), so fungal heterogeneity is unlikely to explain the observed high level of cyanobacterial diversity. However, in contrast to other tripartite *Peltigera* species, the cephalodia of *P. venosa* are located on the veins of the lower surface of the thallus and may exhibit different degrees of lichenization, ranging from loosely associated colonies to well corticated cephalodia (**118**). Further studies on *P. venosa* should include examination of the degree of cortication of cephalodia and heterocyst frequencies as well as genetic identification.

The *Nostoc*-bryophyte (*Anthoceros* and *Blasia*) associations contained a similar level of diversity as was found in *P. venosa* where different symbiotic colonies of one thallus may or may not contain the same intron sequence (Fig. **1c**). In this case, however, all sequences came from *Nostoc* cells within the bryophyte cavities so that at least the degree of structural association was the same. These results are perhaps not surprising in light of previous infection studies where many different *Nostoc* strains were found to infect axenically grown bryophytes (**35**). In these associations, it is only the gametophyte that is infected (**144**), and each new generation thus requires a new infection (though asexual dispersal units containing cyanobacteria have been reported for *Blasia pusilla* [**34**]). Variation in one thallus may depend on multiple strains infecting the young gametophyte or additional strains entering the thallus from the surrounding.

The different general patterns between lichens (papers I–III), bryophytes (paper IV) and cycads (**29**) are thus that lichens contain a single *Nostoc* in each individual (or thallus) whereas bryophytes and cycads may contain different *Nostoc*s in different cavities or coralloid roots respectively. This was shown using one and the same approach: the nucleotide sequence of the $tRNA^{Leu}_{UAA}$ intron. In general, different genetic markers and fingerprinting methods with different resolution have been applied in different studies concerning the diversity of cyanobacterial symbionts. The material may also be from the field, from botanical gardens or isolated strains. This makes comparisons of results from different studies and differences between the symbiotic systems hard to evaluate. Comparisons between different symbioses can only be made when the biological material is equivalent (e.g., field material) and the methods are comparable.

Patterns of Specificity

When the examination of lichen symbionts is applied to different species from a single site and the same species from different sites within the same area, the general pattern is that lichen species rather than field site is important for the identity of the *Nostoc* symbiont. This was noted in mixed collections where two different lichen species growing in physical contact with each other contained different *Nostoc* sequences, but the different sequences could be found in the corresponding lichen species from other locations (Fig. 6d). Some patterns were even found when lichens from remote geographic areas were investigated (Fig. 6e). Lichens *P. aphthosa*, *P. canina* and *N. arcticum* all had different *Nostoc* symbionts with identical sequences in samples of one species collected in both Sweden and Finland. When the same lichen species were collected from Europe and North America, identical sequences were found in *P. membranacea*, closely related sequences in *N. resupinatum* but different sequences in *P. neopolydactyla*.

Even though the general pattern is that it is the lichen species rather than the collecting site which is important for cyanobiont identity, there is not only one *Nostoc* for each lichen species. In several of the species, two different *Nostoc* sequences were obtained from different samples. In *P. aphthosa*, for example, the same two different sequences were found in different samples from both Finland and Sweden. In *P. membranacea*, identical sequences were found in samples from Sweden and USA, but one sample from USA contained three deviating nucleotides. In a different study based on 16S rRNA gene sequences, Miao et al. (98) showed that different color morphs of lichen *P. membranacea* contained different *Nostoc* strains. *Peltigera neopolydactyla,* on the other hand, exhibited a greater diversity of *Nostoc* symbionts in samples collected both in Finland and USA. This species is taxonomically difficult, and many different types exist (52, 163). Some correlation was found between variation in secondary chemistry, which are important characters in lichen taxonomy, and *Nostoc* symbiont in this species, but further studies are needed to verify such correlations.

In addition to the spatial stability of the *Nostoc* symbiont of several lichen species, temporal stability was shown for the lichen *P. aphthosa*. Identical sequences were detected in one population of this species collected five years apart.

That different lichen species growing in physical contact with each other contain different *Nostoc* strains indicates that the lack of variation seen in lichens is not caused by a corresponding lack of variation at the site where

the lichen grows. In fact, from one single site (about 1m^2) in Oregon (USA), 11 clearly distinct *Nostoc* sequences (5 from different symbiotic associations and 7 from free-living colonies or soil samples) and an additional 3 cyanobacterial subsection IV sequences that may be more closely related to *Anabaena* strains were amplified (unpublished). One of the sequences from the soil was identical to a *P. venosa* sequence, but as discussed above, the true symbiotic nature of some of these strains could be questioned. The other six free-living strains were unique. The diversity in the field is an interesting and important future area of investigations. This pool of diversity in the habitats where organized lichen thalli develop highlights the biological significance of the lack of variation encountered in the lichen specimens.

Hypotheses for Patterns of Specificity

That identical intron sequences are found in a specific lichen species from different remote areas and that a large diversity of different sequences are found in one field site raises the question of spread of these lichens. There are two possibilities for these patterns: 1) the fungus is specific in recognizing and selecting only a certain *Nostoc* from the diverse soil community or, alternatively, 2) the lichens exhibiting these geographical patterns primarily spread as the holobiont, with propagules containig both symbiotic partners. Many lichens are known to spread using this strategy, but the relative importance of different dispersal strategies is hard to elucidate (70). The first option would require that the same *Nostoc* is available at different field sites and in some cases have a global distribution. Global distribution of cyanobacteria on the level of botanical species is common, also for the genus *Nostoc* (33, 174). This option would also require a very specific signalling to differentiate between different *Nostoc*s with very similar physiological capabilities. The second option does not require complex signalling or global distribution of free-living *Nostoc*s, but instead that the lichenized association (for the species exhibiting geographical patterns in photobiont identity) is more close and long-term than generally believed. When a lichen is formed, the connection between the two symbionts may last for long periods, and a successful match between fungal and cyanobacterial genotypes may stay associated and effectively spread as symbiotic propagules over vast areas. The species used in this thesis generally do not contain isidia or soredia (163), and so fragmentation would be the holobiont propagule. For the species not showing the same kind of geographical patterns in cyanobiont identity, a different ecological strategy may well be the reason. *P. neopolydactyla,* for

example, may be more prone to disperse via sexual spores and associate with new photobionts. The taxonomic situation in this species with a variable morphology and chemistry (**52, 65, 163**) may support this. Using a similar approach, Oksanen et al. (**2001**, unpublished) found that certain old-growth associated epiphytic lichens which appear to form a functional guild (**141**) share the same or very similar photobiont strains. Thus, these lichens seem to exhibit a different pattern of cyanobiont specificity from those found in this study.

This emphasizes the complexity of the intersymbiotic relations in lichens where different patterns and processes are important in different lichen species.

Different Physiological Types

Mycobionts of cephalodiate lichens may sometimes produce different morphotypes in symbioses with green algal and cyanobacterial photobionts (**6, 50**) . These morphotypes can live separate lives, but may also in rare cases combine into one thallus. Such chimeroid lichens, which contain green algae and cyanobacteria as photosynthetic components in different parts of a single thallus, are called photosymbiodemes (**20, 67, 71, 158**).

When examining a *P. aphthosa* photosymbiodeme containing both bi- and tri-partite cephalodiate thalli, identical *Nostoc* sequences were found in both thallus types (Fig. 6f). This suggests that the same *Nostoc* strain is capable of both symbiotic roles found in the respective thallus types. In bipartite lichens, it functions mainly in photosynthesis, but also provides some nitrogen to the host; heterocyst frequencies are as in free-living strains (**132**). In tripartite associations on the other hand, heterocyst frequencies are highly increased and nitrogen fixation is the primary function of the cyanobiont (**132**). This indicates that different physiological roles of the *Nostoc* symbiont in different systems is not dependent on different types of *Nostoc* strains. The potential of a single *Nostoc* to function in different types of symbioses as also found from reinfection studies (**35, 74**) highlights the impressive symbiotic abilities of these microorganisms.

Diversity and the species concept

The aim of this project was not to resolve phylogenetic relations within *Nostoc* or set up criteria for species delimitations. However, the need for such a framework became evident as patterns in sequence variation in lichen cyanobionts were revealed. The many existing botanical species (**46, 85**) as

well as the form species (**170, 174**) must be evaluated using a polyphasic approach (**47**) in order to obtain a biological framework into which studies such as this can be incorporated. These patterns of specificity found in lichens are now only defined in terms of intron sequence.

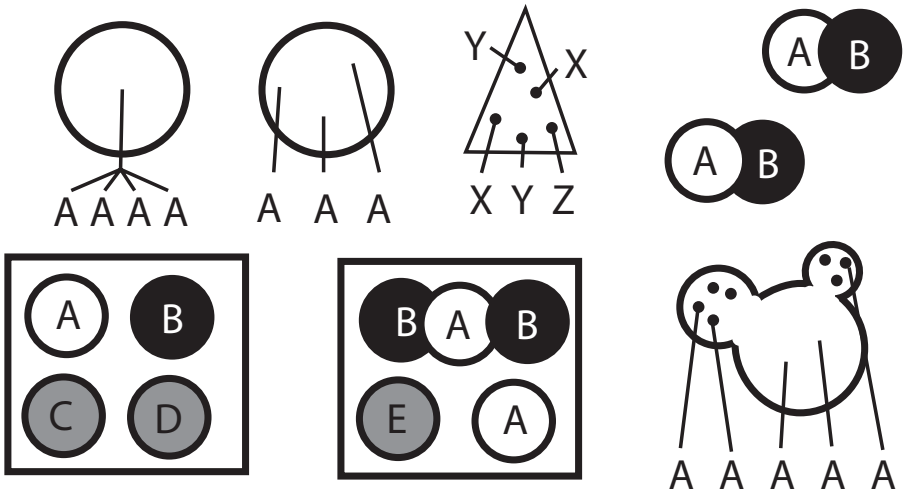


FIG 6. Summary of the results on diversity and specificity of symbiotic *Nostoc*. Circles represent lichen thalli with different shading indicating different species. The triangle represents a bryophyte thallus. The different letters represent different *Nostoc* sequences. Squares represent distant collecting sites (Sweden/Finland/USA).
a) The PCR product from one spot on a thallus consistently contained only one sequence implicating that the in situ symbiont consists of a single strain rather than a community of different *Nostoc* strains.
b) Each lichen thallus contained a single sequence implicating that the fungus grows together with its symbiont as the thallus grows rather than obtaining new strains from the free-living community.
c) In the bryophyte association, more diversity is found; different colonies of a single gametophyte may contain the same or different sequences.
d) The identity of the *Nostoc* symbiont in the lichens used in this thesis is more restricted by the lichen species than the site where it grows.
e) Patterns of diversity are found on geographical and global scales, but different lichen species seem to vary in *Nostoc* diversity.
f) Identical sequences are found in bipartite and tripartite lobes of photosymbiodemes implicating that one *Nostoc* strain is capable of performing the physiological functions of both bipartite and tripartite lichens.

CYANOBIONT SPECIFICITY TESTED IN FIELD INOCULATIONS (PAPER V)

The stability of the association between *Nostoc* and fungal host was addressed by attempting to introduce cyanobacterial strains into established lichen thalli of the species *Peltigera aphthosa*. As previously described, low cyanobiont diversity was found in this species and the same two *Nostoc* sequences were found in different samples from both Sweden and Finland.

In three different populations of *P. aphthosa* growing in the field, each consisting of seven thalli, all visible cephalodia were experimentally removed. The manipulated lichens were inoculated with known strains of cultured cyanobacteria and left to develop new cephalodia. Out of the seven cyanobacterial strains used, two were common laboratory strains, and five were isolated from lichens. After a summer in the field the identities of cyanobacterial symbionts in 80 newly formed cephalodia were determined by sequencing the $tRNA^{Leu}_{UAA}$ intron. All epiphytic colonies of free-living cyanobacteria were also analyzed. It was shown that foreign cyanobacteria are not readily incorporated into established *P. aphthosa* thalli. All newly formed cephalodia contained the same intron sequence, this being 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. Finding these strains growing epiphytically shows that symbiotically competent strains can survive in the field. Even though it is generally assumed that strains with symbiotic competence also are occuring as free-living, their survival in the field has never previously been shown.

This study shows that it is possible to monitor the growth of different cyanobacterial strains over longer periods in the field. Strains can be released into natural habitats, alone or in mixes, and their survival and success monitored continuously. A similar approach, showing survival of mycobiont transplants, has recently been performed (38). These kinds of studies will help identify traits needed for success in natural habitats. In studies of symbioses, the approach can be very fruitful, and similar experiments should be attempted in other symbiotic systems where lack of specificity has been shown in reinfection studies.

This study (paper V), together with our previous findings (paper I–III), 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 rather unspecific.

EVOLUTIONARY PATTERNS IN THE tRNA$^{LEU}_{UAA}$ INTRON (PAPER VI)

The molecular marker used when investigating diversity and specificity in cyanobacterial symbioses was the tRNA$^{Leu}_{UAA}$ intron. This marker is variable on a subgeneric level in cyanobacteria and has previously been used in studies on taxonomy and population structure in plants (**40, 48, 49, 154,**).

From our studies, we obtained a unique set of closely related tRNA$^{Leu}_{UAA}$ intron sequences from the genus *Nostoc*. Using these sequences, together with other cyanobacterial tRNA$^{Leu}_{UAA}$ intron sequences from the databases, we analyzed evolutionary patterns in this genetic marker. The variation between different introns is not randomly distributed, but strongly restricted by the secondary and tertiary structure of the intron. The evolutionary forces working on this intron may include the following:

–nonrandom mutations (in part caused by lack of base–pairing in single-stranded DNA structures during transcription and replication) (**173**)

–slipped strand mispairing of repetitive regions (**54, 160**)

–homologous recombination

However, phylogenetic analyses using conserved and alignable parts of the intron grouped closely related sequences generally consistent with the corresponding **16**S rRNA gene analyses. Conserved elements in the intron could thus be used for identification at the genus level and more variable regions for the identification of different *Nostoc* strains.These results are in agreement with an evolutionarily old intron that is not laterally mobile (**14, 124**). However, Rudi et al. (**145, 146**) have suggested a more complex evolutionary history for this intron. Possible explanations for their interpretations are presented in Paper VI.

All *Nostoc* sequences share a high degree of similarity, except in one stem-loop where both sequence and size variation is considerable (Fig. **7**). Based on sequence similarity, this stemloop could be divided into two classes. In each class, the stem-loop was found to be built from two base pairing heptanucleotide repeats. Size variation was primarily due to different numbers of repeats, possibly caused by slipped strand mispairing during replication. Some strains also contained additional sequences in this stem-loop not following the heptanucleotide repeat motif. A search in the genome sequence of *Nostoc punctiforme* identified several loci showing similarity to

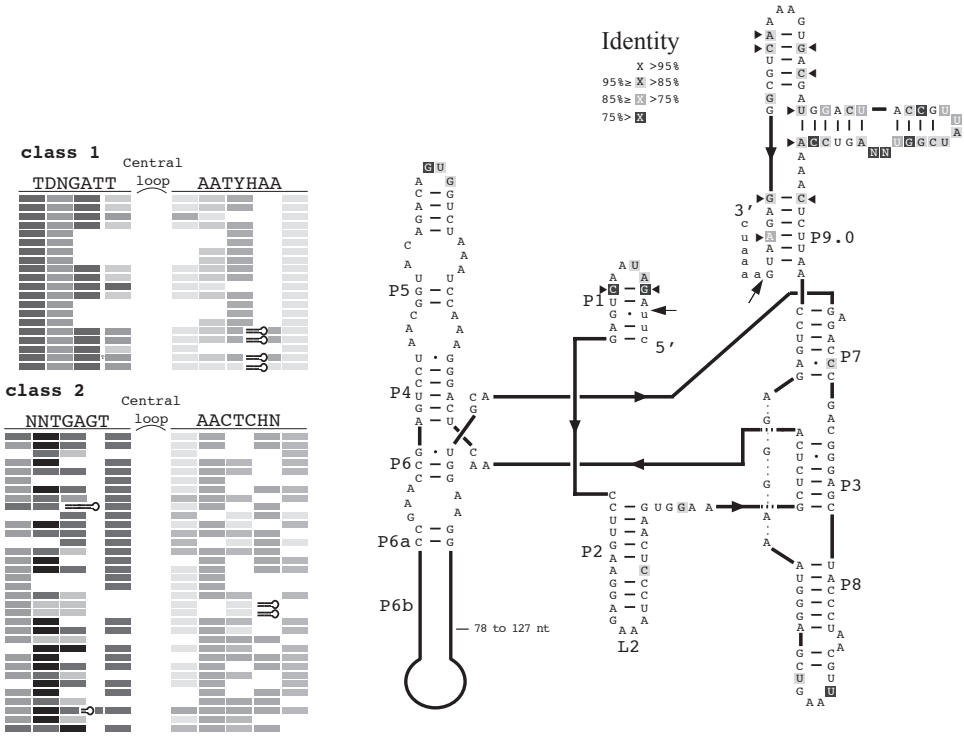


FIG. 7. Consensus sequence of the $tRNA^{Leu}_{UAA}$ intron from *Nostoc* represented in a two dimensional model where base pairing regions are shown (**27**). The degree of variability in positions are seen from the degree of shading. Size variation is restricted to one region (P6b) which could be divided into either of two classes based on nucleotide sequence (class **1** and **2**). The sequences observed in both classes could be divided into repeats of seven nucleotides (represented as boxes with the consensus sequence of each repeat indicated above). Different shades represents different varieties of the repeat. Size variation was in most cases caused by different numbers of repeats. In some sequences, stretches of DNA not conforming to the heptanucleotide repeat motif were found (represented as hairpins).

these additional sequences. Furthermore, the regions flanking these loci contained the same, or similar, heptanucleotide repeats as those flanking the corresponding sequences in the intron. Homologous recombination between different loci in the genome containing the same, or similar, heptanucleotide repeat motifs may have given rise to these sequences in the intron.

The same situation with variation caused by several processes other than random mutations are also found in other RNA molecules often used in similar studies such as the 16S rDNA and ITS regions (18, 54, 69, 159). These types of evolutionary processes are crucial to consider when using a molecular marker in ecological or systematic studies, especially when phylogenetic reconstructions are attempted.

DIFFERENTIAL PROTEIN EXPRESSION IN LICHENIZED AND FREE-LIVING NOSTOC (PAPER VII)

Functional genomics of lichen symbionts is a new field that has great potential to increase our knowledge of the important processes and modifications occuring in the symbiotic state. As established in our previous studies, there is only a single *Nostoc* strain in each thallus or population for the examined lichen species. This is important if any type of molecular or biochemical experiments depending on sequence information is used. Another important factor is that *Nostoc* symbionts in these lichens are closely related to *N. punctiforme* ATCC 29133 (see paper VI) for which the genomic sequence is in the finnishing phase and much physiological knowledge is available (96). This strain is a deposition of the Pasteur Culture Collection strain PCC 73102, which was originally isolated from a symbiotic association with a cycad. By identifying differences in the expression of proteins between symbiotic and free-living conditions, it may be possible to understand the metabolic and developmental settings in the symbiosis. Analyzing such differences was done using 2 dimensional gel electrophoresis (2DE). Putatively interesting proteins were analyzed using mass spectroscopy (MS). The peptide sequences obtained from this analysis were used in searches in GenBank and the *Nostoc* genome sequence. By cleaving the interesting protein, several peptides from single proteins were obtained which increases the reliability of the identification.

In order to obtain a protein extract from the *Nostoc* symbiont of a lichen, the cyanbiont was separated from the fungal partner. This was done in a series of steps, involving homogenization, centrifugations and two-phase

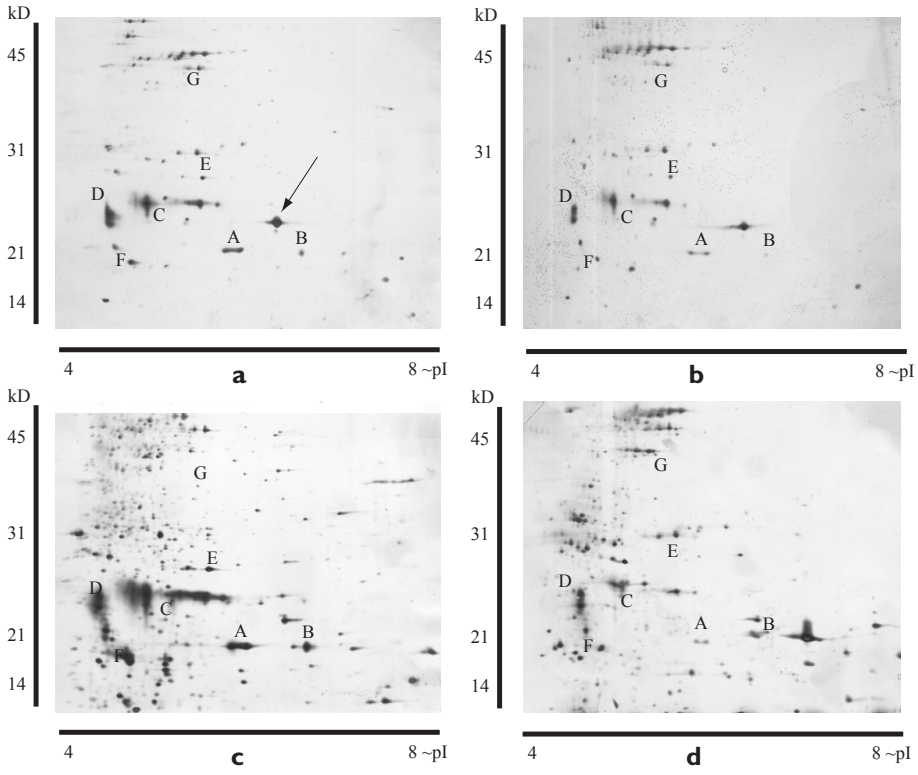


FIG. 8. Silverstained 2D gels of the soluble protein fraction of different symbiotic and free-living *Nostocs*. Letters in the gels are included for orientation. The protein labelled with an arrow has been partially sequenced and the corresponding ORF in the *Nostoc punctiforme* genome identified.

a–b) 2D gel of soluble protein extract of cyanobionts from active (a) and dormant (b) bipartite lichen *Peltigera membranacea*. One population was harvested in the winter. Half of the thalli were incubated in room temperature for one week with daily watering (a) and the other half was kept frozen (b) until the cyanobacterial symbiont was purified and analyzed.

c) 2D gel of soluble protein extract from a free-living *Nostoc* isolate from lichen *P. membranacea* (from the population as used above).

d) 2D gel of soluble protein extract from the cyanobiont of the tripartite lichen *P. aphthosa*.

separation that, based on density differences between cyanobacterial cells and the fungal hyphae, yields a fairly pure cyanobacterial fraction (**167**). The cyanobacterial cells remained viable during the purification and the cyanobiont fraction could be obtained in about three hours from first crushing the lichen.

Shown in Figure 8 are 2D gels of *Nostoc* extracts from different free-living and symbiotic states.

Comparing freshly isolated cyanobionts from metabolically active and dormant thalli reveals no major differences (Fig. 8a and 8b). This is in agreement with the nature of lichens where metabolic activity is resumed as soon as water status, temperature and light allows for photosynthesis (**81, 108**). This makes it possible for lichens to efficiently take advantage of climatic fluctuations, for example in boreal areas during warm days in spring when plants are still dormant (**141**). This stability in major protein content could also indicate that transcriptional activation may not be necessary for activity which in turn could explain problems with RNA work on lichen cyanobionts (unpublished).

Comparing these patterns to that of the free-living culture of the *Nostoc* symbiont from this lichen (Fig. 8c) reveals several differences. Analyzing these differences will provide insight into the differences in *Nostoc* metabolism found in a lichen thallus and free-living. The protein indicated by an arrow in figure 8a, is one of the most prominent proteins of the lichen cyanobiont, but is expessed at a lower level in the isolated symbiont from this lichen (Fig 8c). No detectable protein was found in this position on gels with extracts from free-living *N. punctiforme* (see paper VII). This protein was analyzed using MS and the corresponding ORF from the *N. punctiforme* genome shows homology to fasciclin domain containing proteins. These are all surface associated proteins involved in cell contact. Among the most homologous sequences were a symbiotically induced gene from *Rhizobium* (**116**) as well as a symbiotically induced gene from the marine cnidarian *Anthopleura* living in symbiosis with algae (**139**). The function of these proteins in symbiosis is not known, but a *Rhizobium* mutant deficient in this protein, shows an intermediate phenotype where symbiotic efficiency is decreased but not completely lost (**116**).

The cyanobiont of cephalodiate lichens can be purified the same way as the symbiont from bipartite lichens. However, the low percentage of cyanobacterial cells in these thalli leads to a drastic increase in the number of thalli needed for extraction. In figure 9 d, the protein pattern from the cyanobiont of *P. aphthosa* is shown. Differences between the physiological roles of the

cyanobionts in bi- and tri-partite species may be investigated comparing this pattern to that found in bipartite cyanobionts (Fig. 8a).

Differences in major proteins that can be found using this approach can give information which will allow for speculation on the upstream developmental signals and pathways important for the symbiotic growth state. This will make it possible to make educated guesses about which genetic switches may be important in symbiosis and further investigate these in future studies by looking at RNA expression or reinfection experiments using mutants.

PERSPECTIVES

The focus of this thesis is *Nostoc* as a symbiont in lichens. However, as noted earlier, *Nostoc* forms endophytic symbiotic associations with a wide range of organisms, and is also involved in many less organised associations (**1, 103**). Why is it that this specific cyanobacterial genus shows such symbiosis competence, and what may it gain from this? In all cases, *Nostoc* gains an additional niche which it can fill. Three main advatages could be:

1) Many symbiotic associations may be long term, and thus function as reservoirs for free-living strains. Cycads and lichens are examples of hosts wich can reach considerable age and thus function as reservoirs for *Nostoc*.

2) The host may provide shelter from environmental stress. In association with plants, *Nostoc* can benefit from the ability of the plant to obtain water also when the soil surface is dry therby staying active when free-living strains must rest. In the case of lichens, survival may be improved by obtaining UV protection from fungal pigments and desication protection from the slower and more controlled drying of the lichen thallus. Both desication tolerance and UV protection are common features of *Nostoc* colonies in for example *Nostoc commune*. However, the *wsp* gene, encoding the water stress protein which is crucial for desication tolerance, seems to be missing in the genome sequence of the symbiotically competent *Nostoc punctiforme* (**174**).

3) The host may provide a physical room where there is no competition for space. In the case of lichens, the fungus also ensures that *Nostoc* has access to sun light and thus is able to photosynthesise. Terrestrial *Nostoc* often phase competition from bryophytes, algae and other ground vegetation.

For the host on the other hand, it is of course *Nostoc*'s capability of photosynthesis, N_2-fixation or both that is interesting to benefit from. The mechanisms involved in the release of the products from one or both of these processes are not known, and the extent to which specific symbiotic developmental alternatives and transport systems are used can only be speculated upon.

The best studied N_2-fixing symbiotic system is that between *Rhizobium* and Leguminous plants (**53**). This association involves specific communication between the partners with signaling in several steps resulting in com-

plex structural modifications. Comparisons between this system and cyanobacterial symbioses have been made regularly and homologues to the symbiosis specific genes and proteins have been studied (**136, 162**). However, at least for the *Nostoc* homologue to *nodM*, the function seems not to be related to symbiosis (**162**). If the range of symbiotic hosts, structures and metabolic functions are considered (table **1**), it seems likely that the *Nostoc* containing symbioses are the consequence of the exploitation of natural features of *Nostoc* rather than one, or several, specific symbiotic developmental alternative(s) controled by one/several specific signalling pathway(s). The high level of cross infectivity between symbionts from different systems (**35, 74**) also shows that there are not generally separate *Nostoc* lineages in the different systems which could have allowed for coevolution of specific signalling pathways in certain host/symbiont pairs. Some features that together may lead to the suitability of *Nostoc* as a symbiont could be:

– Chemotactic motility of hormogonia making it possible for a host to obtain the cyanobacterium from the surrounding soil. As argued earlier, this feature may not be so important in lichens, but is vital in associations with plants (**1**).

– Metabolic flexibility provides transport systems and flexibility which could be exploited in symbiosis.

– Translocation to the extracellular space of a large fraction of the resources in free-living *Nostoc*s is, in some way, equivalent to the symbiotic state.

– *Nostoc* is already similar to a symbiotic system in that cells of the filament with different functions (even though of the same organism) exchange metabolites.

This division of labour in the filament is perhaps the most important feature. The role of the heterocyst is of fundamental importance in most symbiotic systems (table **1**). The heterocyst is a developmental end point that will never divide or further differentiate. The function of the heterocyst is thus N_2-fixation. Because there are no developmental alternatives for the heterocyst, it seems likely that the factors controling the rates of N_2-fixation are the availability of reductant and other necessary compounds. In the free-living state, this would be controled by the vegetative cells, which in turn have the possibility to partition its resources in the form of photosynthate between nitrogen fixation, cell-division or development of hormogonia. Host interaction with this communication between the different cell types may provide possibilities to exploit either of the valuable traits of this bacterium.

If the transfer of reductant and energy are the regulatory factors in N_2-fixation, the host can provide these substances and thereby increase N_2-fixation. If the host simultanously physically restricts growth of the filament, the provided energy can not be used for vegetative growth.

One aspect of the cyanobacterial filament which may be fruitfull to further investigate is the periplasmic space. The precise routes for metabolite exchange in the filament have not been determined, but one hypothesis states that the transfer of combined nitrogen takes place in the periplasmic space (which is believed to be continuous between heterocysts) (**105**). Whether also the photosynthate could be transported this way in the opposite direction is not known, but as far as I can see possible. Another important substance believed to be localized to the periplasmic space is PatS which has been studied in the free-living strain *Anabaena* PCC **7120**. This **13**, or **17**, amino-acid peptide inhibits heterocyst formation (even when added exogenously), and is thus crucial for heterocyst spacing pattern (**178**). A *patS*$^-$ mutant has a phenotype with abnormally short intervals between heterocysts (high heterocyst frequencies) and contiguous heterocysts (**179**) similar to the observed pattern in many symbiotic associations. Host control over the periplasmic space could thus lead to increased heterocyst frequency and the continuous activity of these heterocysts could be ensured by exogenously provided photosynthate to the periplasm. The products of N_2-fixation could also be harvested from this space. However, in most cases it seems as if NH_4^+ is the nitrogen species transferred from the cyanobiont in the symbiotic state (**1, 133**). Low levels of glutamine synthetase (GS), the primary NH_4^+ assimilating enzyme in heterocycsts, has been suggested as the mechanism (**76, 125, 132**). One possible explanation for the release of NH_4^+ in the symbiotic state consistent with the model suggested above would be that periplasmic transport of glutamate from vegetative cells to heterocysts is affected by the host. This glutamate is, together with NH_4^+, the substrates for GS and lack of glutamate in heterocysts may allow for NH_4^+ to be released. Glutamate used for glutamine synthesis in the heterocyst is believed to primarilly be obtained from vegetative cells since Glutamate synthase (the key glutamate producing enzyme) levels in heterocysts are negligable (**94**).

The normal level of heterocysts in bipartite lichens could be explained by a lack of exogenously provided photosynthate from the host. Consistent with this is that prolonged growth of the *patS*$^-$ mutant leads, at least partially, to a restoration of the wild type phenotype probably as a consequence of the need for a balance between photosynthesis and N_2-fixation (**179**).

There is no experimental support for this model, but it provides a simple hypothesis which can be tested in future experiments.

I have only started investigating one factor important in this model, the outer membrane. This membrane is the barrier between the periplasmic space and the host. Very little is known about this membrane in cyanobacteria in general, and in *Nostoc* in particular 64. A major component of the outer membrane are the porins. I have isolated several putative porin encoding genes from a genomic *Nostoc* library, and later from the *Nostoc punctiforme* genomic sequence, using the unique peptidoglycan binding motif found in cyanobacterial porins (55). Further characterization of the expression of these proteins and the status of the outer membrane in the symbiotic state could help understand the role of the periplasmic space. There is one interesting connection between this hypothesis and the fasciclin homologue shown to be highly expressed in the symbiotic state (paper VII). When the mycobacterial homologue to this fasciclin protein, the major antigen MPB70, is expressed in *E. coli*, the protein is exported over the cytoplasmic membrane as expected. However, the protein does not only remain in the periplasmic space, but also cross the outer membrane. Also other periplasmic proteins, such as beta-lactamase, could be recovered from the growth medium when MPB70 is expressed in the cell. It was suggested that MPB70 interact with the outer membrane in a nonspecific way through hydrophobic interaction which causes this leakage (60). This kind of leaky outer membrane could be reasonable in both pathogenicity and symbiosis and would nicely fit in the model proposed above.

CONCLUSIONS

* The tRNA$^{Leu}_{UAA}$ intron is an operational marker for subgenus identification of cyanobacterial strains.
* Only one *Nostoc*, rather than a community of strains, is found in each point/cavity/cephalodium in lichen- and bryophyte associations.
* There is generally only one strain in each lichen thallus, but often several strains in different bryophyte cavities of one thallus.
* The identity of the *Nostoc* symbiont is more dependent on lichen species than collection site for the species investigated. The same seems not to be true for *Nostoc*-bryophyte associations.
* Patterns in the identity of the cyanobiont can for some lichen species be found over great distances.
* The same *Nostoc* can be modified by the fungal host to perform the two different functions found in bipartite and tripartite associations.
* Free-living *Nostoc* strains isolated from different lichen species are not readily incorporated into established thalli of *Peltigera aphthosa*.
* Conserved parts of the tRNA$^{Leu}_{UAA}$ intron could be used for identification of *Nostoc* on a genus level, and variable regions, made up of variable numbers of heptanucleotide repeats, for the identification of specific *Nostoc* strains.
* Evolutionary patterns in genetic markers are important to consider when nucleotide variation is analysed in a biological context.
* There are differences in the repertoire of proteins which are expressed in *Nostoc* under lichenized and nonlichenized conditions. These differences may provide valuable insight into modifications required for symbiotic competence.
* One of the most highly expressed proteins in *Nostoc* symbionts of lichens is a homologue of symbiotically induced genes from other symbiotic systems.

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