Arch Microbiol (1987) 148:172–177

Archives of
Microbiology

Isolation and characterization of a temperate cyanophage for a tropical *Anabaena* strain

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Abstract. In this paper we describe the isolation and characterization of a temperate cyanophage N(S)1 of the genus cyanopodovirus which produces turbid plaques on the host *Anabaena* 77S15 isolated from tropical soil. Its properties have been compared to those of other well-characterized cyanophages. In addition, two strains of *Anabaena* 77S15 lysogenic for N(S)1 were isolated. N(S)1 seems to be integrated into the chromosome of the two lysogens, and a 2 kb plasmid present at a low copy number in the non-lysogenic strain is amplified significantly.

Key words: Cyanophage – Cyanopodovirus – Cyanobacterium – *Anabaena* – *Nostoc* – Lysogen

The first virulent cyanophage for a heterocyst forming filamentous cyanobacterium was isolated in 1971 by Adolph and Haselkorn (1971). According to the generic assignment of the host, *Nostoc muscorum* (PCC7120), the phage was called N1. However, the host strain was recently classified as an *Anabaena* by Rippka et al. (1979). Subsequently, some twenty cyanophages capable of infecting cyanobacteria of the genera *Anabaena* and *Nostoc* have been reported and characterized (Koz'yakov 1977; Hu et al. 1981). According to the nomenclature of Safferman et al. (1983), these cyanophages belong either to the genus cyanopodovirus, characterized by a short tail, or to the genus cyanomyovirus characterized by a long contractile tail. Among these cyanophages, one was found to be temperate (Khudyakov and Gromov 1973), but to our knowledge transduction experiments have not yet been reported.

In this paper we describe the isolation of a cyanophage, N(S)1, which induces turbid plaques on a strain of *Anabaena* isolated from tropical soil (Franche and Reynaud 1986). The properties of this phage were examined and compared to other cyanophages which attack heterocystous cyanobacteria assignable to the genera *Anabaena*/*Nostoc*. Furthermore, lysogens for N(S)1 have been isolated and characterized.

Material and methods

Biological material

The PCC strains listed in Table 1 and *Anabaena azollae* var. *filiculoides* isolated by Tel-Or et al. (1983) were obtained from the Pasteur Culture Collection (Rippka et al. 1979), Institut Pasteur, Paris, France. The tropical *Anabaena* and *Nostoc* (strains 74S08 to 79S03 and 74S04 to 79S04) were described by Franche and Reynaud (1986). The cyanophage N1 isolated by Adolph and Haselkorn (1971) was kindly provided by R. Rippka.

Cyanobacterial cultures

Cultures were maintained on slants of BG-11$_0$ medium (Rippka et al. 1979). For analysis samples were grown in 100 ml of BG-11 medium in 250 ml flasks incubated at 30° C and exposed to a light intensity of 500 lux. Before each experiment, the absence of contaminating bacteria was checked according to the procedure of Rippka et al. (1979).

Isolation of cyanophages

Water samples were collected from ponds, streams and rice fields in Senegal. After passage through Millipore filters (type HA, 0.45 µm pore size), aliquots of 1 ml were assayed for plaque formation (see below) on the tropical *Anabaena* and *Nostoc* strains listed in Table 1. After 2 weeks of incubation in the light, zones of lysis were picked, resuspended in 1 ml of BG-11 medium (Rippka et al. 1979) and a Millipore filtrate was produced as described above. The cyanophage was considered to be purified after three consecutive single plaque isolations.

Plaque assay and host-range study

The plaque assay was similar to that described previously for the cyanophage LPP-1 (Luftig and Haselkorn 1967). The host-range was determined by spotting 50 µl of a phage stock solution at a concentration of 2×10^8 plaque forming units per ml (PFU/ml) onto lawns of cyanobacteria containing 10^8 cells in a 0.5% agar overlay. The plates were examined after 10 days of incubation at 30° C in the light.

Phage stock preparation

The cyanobacterial host (*Anabaena* 77S15) cultivated in 100 ml BG-11 medium for 2 weeks yielded about 2×10^8 cells/ml. Cyanophages were then added to obtain a multiplicity of infection of 0.01 PFU/cell. After 10–15 days, 1 ml of chloroform was added and the cell debris was removed by centrifugation. In general, titers of 2×10^9 PFU/ml were obtained.

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Table 1. Filamentous heterocystous cyanobacteria examined for susceptibility to cyanophage N(S)1

Genus	Strains	Reference
Anabaena	PCC7120, PCC7122	Rippka et al. 1979
	74S08[a], 74S12[a], 74S18[a], 74S19[a], 74S23[a], 74S24[a], 74S25[a], 74S26[a], 75S03[a], 75S19[a], *77S15*[a], 77S19[a], 79S01[a], 79S02[a], 79S03[a]	Franche and Reynaud 1986
	Anabaena azollae var. *filiculoides*	Tel-Or et al. 1983
Nostoc	PCC73102, PCC 6720	Rippka et al. 1979
	74S04[a], 74S06[a], 74S07[a], 74S09[a], 74S22[a], 74S29[a], 74S51[a], 74S53[a], 74S54[a], 74S56[a], 74S60[a], 74S66[a], 77S17[a], 79S04[a], 79S05[a]	Franche and Reynaud 1986
Scytonema	PCC7110	Rippka et al. 1979
Calothrix	PCC7102	Rippka et al. 1979
Fischerella	PCC7414	Rippka et al. 1979

Electron microscopy of phage particles

A phage stock was concentrated to $10^{11}-10^{12}$ particles per ml with polyethylene glycol 6000 (Elmerich et al. 1982). A drop of this suspension was applied to a 200-mesh copper grid coated with carbon and stained with 2% uranyl acetate. The grid was examined using a Siemens Elmiskop 101 electron microscope.

Adsorption and one-step growth experiments

An adsorption experiment was performed according to the method described for cyanophage N1 by Currier and Wolk (1979). For the one-step growth experiment, the cyanobacterial culture (2×10^7 cells/ml) was treated for 30 s with a homogenizer (Ultra Turrax, Bioblock) to fragment the filaments into short trichomes (two or three vegetative cells). Phages were then added at a multiplicity of infection of 10^{-2} PFU/cell. After 2 h of incubation in the light aliquots of the culture were withdrawn and diluted 10^{-3}, 10^{-4}, 10^{-5}-fold in 10 ml BG-11 medium. Phage release was followed by titration of the suspensions on overlays of *Anabaena* 77S15 at intervals of 4 h over a period of 6 days.

Effects of temperature, pH and ions

Effects of temperature, pH and ions on the stability of the cyanophage were determined as described by Safferman and Morris (1964).

Preparation of antiserum and serological properties

The antiphage serum and the serum value (K value) were obtained according to Eisenstark (1967). The serological relationship of the cyanophages was determined by the method of Adams (1959).

Isolation of lysogens

An exponentially growing culture of *Anabaena* 77S15 (2×10^7 cells/ml) was infected at a multiplicity of one PFU/cell. After 3 weeks of incubation in the light, the surviving cells were centrifuged, washed three times in BG-11 medium and plated to isolate colonies. The colonies were then tested for their ability to cause lysis on 0.5% agar overlays on the indicator strain *Anabaena* 77S15. Colonies producing a zone of lysis were picked, purified by three consecutive single colony isolation on BG-11 plates and finally grown in liquid medium for 3 weeks. The resulting cultures were then treated for 48 h with antiphage serum to precipitate free N(S)1 particles at a dilution of 1:10, washed three times, diluted 10-fold and cultured for 3 weeks in fresh BG-11 medium. Their supernatant were then checked for the presence of phage particles on overlays of the indicator strain *Anabaena* 77S15.

Mitomycin C treatment

Mitomycin C (Sigma) was added to a final concentration of 1 µg/ml to a young exponentially growing culture of the lysogenic strains (2×10^7 cells/ml). After 24 h, the trichomes were centrifuged and resuspended to the same concentration in fresh BG-11 medium. The culture was incubated in the light for 7–10 days before assaying the supernatant for plaque formation on the indicator strain *Anabaena* 77S15.

DNA isolation procedures

Cyanophage DNA was purified from a 10 l lysate following the procedure of Elmerich et al. (1982). Plamid DNA was prepared according to Humphreys et al. (1975). Total DNA was extracted from vegetative cells by the method of Quiviger et al. (1982) and further purified by caesium chloride-ethidium bromide density gradient centrifugation.

Restriction endonuclease digestions and DNA electrophoresis

Restriction endonucleases (Genofit) were used as recommended by Maniatis et al. (1982). Restriction fragments were separated in 0.7% or 1.5% agarose gels according to Quiviger et al. (1982). Lambda phage DNA fragments were used as molecular weight standards.

Preparation on hybridization probes

Plasmid DNA was labelled with [α-^{32}P]dCTP (400 Ci/mmol, Amersham International) by nick translation (Southern 1975). The specific activity of the labelled DNA was approximately 10^8 cpm/µg of DNA.

Southern hybridization

DNA fragments were transferred from agarose gels onto nitrocellulose filters (Millipore HAWP, 0.45 µm pore size) and hybridized with the heat-denatured probe (2×10^6 cpm/

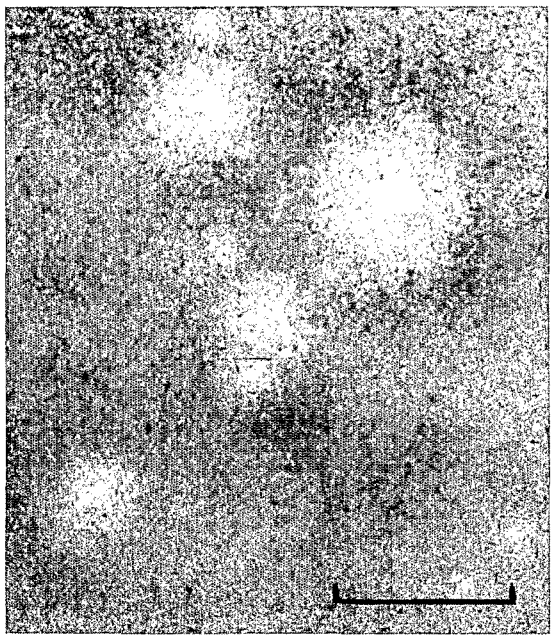

Fig. 1. Plaque morphology of cyanophage N(S)1. The incubation times was 10 days. Bar marker indicates 1 cm

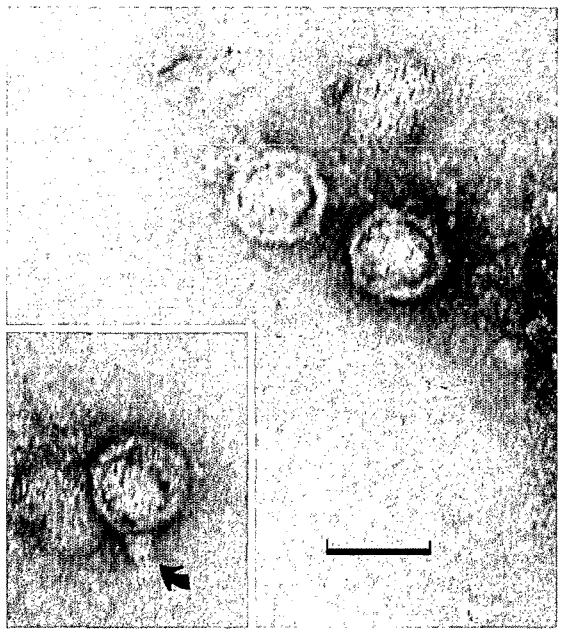

Fig. 2. Electron micrograph of cyanophage N(S)1 negatively stained with uranyl acetate. The *arrow* indicates the short tail. Bar marker indicates 50 nm

slot) following the technique of Southern (1975). Autoradiographs were obtained by exposing the filters for one to several days at −80°C to Kodak X-ray films.

Results

Phage isolation and characterization

Fifty water samples were screened for plaque formation on the thirty tropical *Anabaena* and *Nostoc* strains listed in Table 1. Only one cyanophage, designated N(S)1, was found in a sample collected from a rice field and it appeared to be specific for *Anabaena* strain 77S15, previously identified as *Nostoc muscorum* sp. (Franche 1984). N(S)1 did not infect the PCC strains tested, nor the *Anabaena azollae* var. *filiculoides* (Tel-Or et al. 1983) (Table 1). The host strain for N(S)1, *Anabaena* 77S15, was not sensitive to the cyanophage N1 (Adolph and Haselkorn 1971) (data not shown).

The plaque morphology produced by the cyanophage N(S)1 is shown in Fig. 1. The turbid plaques were 2 to 5 mm in diameter after 10 days of incubation. Heterogeneity in size was preserved even after independent isolation of phage particles from both small and large plaques.

Negatively stained preparations of N(S)1 phages revealed particles possessing a hexagonal head (50 nm) and a short but rarely visible tail of 10 nm (Fig. 2). Double-stranded DNA was extracted from the phage particles purified on caesium-chloride gradients. The molecular weight of the nucleic acid was estimated to be 28 kb (Fig. 3). Linear or circular forms of the DNA were not further investigated.

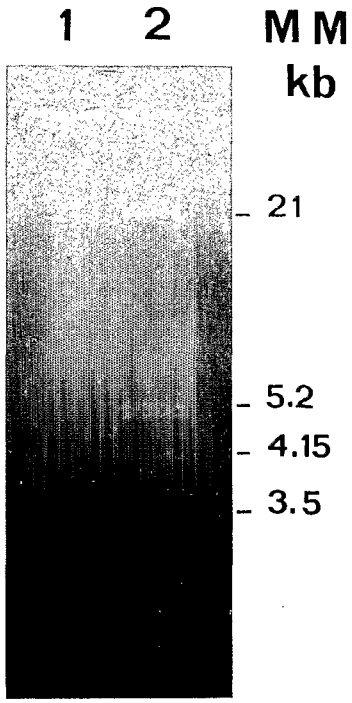

Fig. 3. Agarose gel electrophoresis of DNA extracted from cyanophage N(S)1 (*lane 1*). Molecular weights of phage λ DNA digested by *Hind*III (*lane 2*) are indicate in kilobases

Stability of cyanophage N(S)1

A stock of N(S)1 was stored for 6 months at 4°C without significant loss in titer. Dilution in distilled water did not provoke any loss of infectivity. The same results were obtained after addition of 10^{-3} M EDTA to the phage stock. Thus, divalent cations are not essential to the stability of this phage.

Thermal inactivation tests revealed that N(S)1 was stable up to 65°C. Incubation at 70°C for 60 min resulted in a 40% loss of titer. At 75°C, total inactivation was reached after 20 min (data not shown).

Phage N(S)1 was stable between pH 5 and pH 11. Loss of titer after 30 min of incubation at pH 4 or pH 12 were 40% and 35% respectively. Inactivation was complete after 5 min at pH 2 or pH 13 (data not shown).

Adsorption and one-step growth experiments

90% of the cyanophage were adsorbed on *Anabaena* strain 77S15 after 90 min (data not shown). Sodium citrate (10 mM) inhibited the attachment to the host-cell. The one-step growth experiment revealed that the latent period lasted for approximately 20 h and that the rise period reached a maximum after 48 h. The average burst size was about 70 phage particles per infected cell.

Serological relationship of cyanophage N(S)1 and cyanophage N1

The *K* values of the anti N(S)1 serum neutralization were 110 and 0 for the cyanophages N(S)1 and N1 respectively, indicating that these two phages are not serologically related.

Isolation and characterization of lysogens

Fifty colonies surviving massive infection with N(S)1 were tested for lysogeny on the indicator strain *Anabaena* 77S15. Colonies giving positive results for lysis were restreaked three times on BG-11 plates to obtain single colony isolates before being examined once again for their potential to lyse the indicator strain. After this procedure, 13 colonies were still able to lyse the host. However, after treatment with the N(S)1 anti-serum, only two colonies formed plaques on *Anabaena* 77S15. Supernatants of liquid cultures derived from these two colonies had a phage titer of 10^4 PFU/ml. A second serological treatment confirmed these results. The two lysogenic clones were called *Anabaena* 84LS1 and 84LS2.

After treatment with 1 µg/ml of mitomycin C, the cultures of *Anabaena* 84LS1 and 84LS2 lysed within 7–10 days yielding titers of 10^8 PFU/ml on the host *Anabaena* 77S15. As previously reported (Franche and Reynaud 1986), the host strain 77S15 was also sensitive to mitomycin C. However, electron microscopic examination of the lysates revealed head-like phage particles devoid of nucleic acid and no biological activity could be related to these structures.

To determine whether N(S)1 is being integrated into the chromosome of the lysogens, the extrachromosomal DNA content of these strains as well as that of *Anabaena* 77S15 was examined. No extrachromosomal DNA of a molecular weight equivalent of N(S)1 was found in the lysogens suggesting that integration into the chromosome takes place. However, a rather surprising observation was made: a small circular plasmid of 2 kb was purified from lysogenic strains *Anabaena* 84LS1 (Fig 4a, lanes 1, 2) and *Anabaena* 84LS2 (data not shown). The linear form of the plasmid was clearly observed in total DNA from strain 84LS1 purified by caesium chloride density gradient (Fig. 4a, lanes 3, 5, 6) and in total DNA from strain 84LS2 (data not shown). This plasmid of 2 kb was not detected on agarose gels of DNA isolated from the non-lysogenic strain (Fig. 4a, lanes 7–9).

When the small plasmid of *Anabaena* 84LS2 was isolated and used as the labelled probe for hybridization experiments with total DNA (digested and non-digested) of *Anabaena* 77S15 and undigested DNA of N(S)1, the following observa-

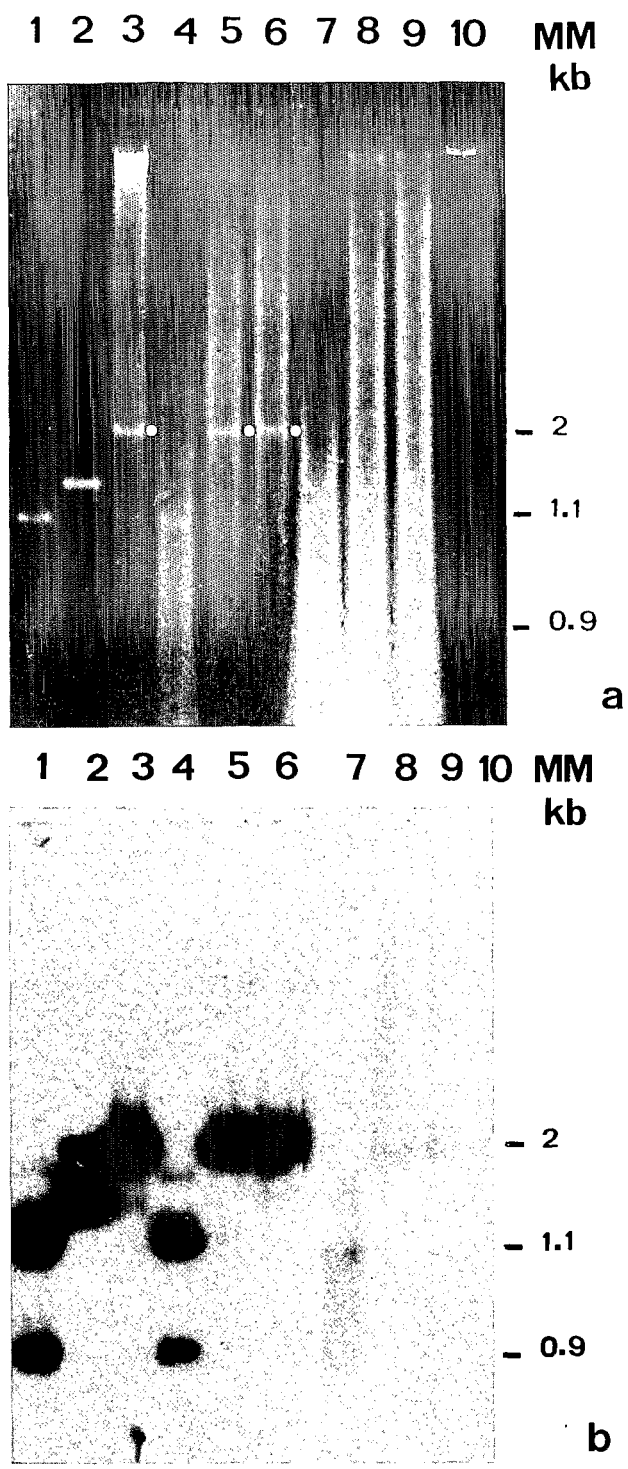

Fig. 4. **a** Agarose gel electrophoresis showing: the 2 kb plasmid of the lysogen 84LS1 digested by *Sau* 3A (*lane 1*), CCC DNA of the same plasmid (undigested) (*lane 2*); total linear DNA of the lysogen 84LS1 (undigested) (*lane 3*) and digested by *Sau* 3A (*lane 4*), *Hind*III (*lane 5*) and *Eco*RI (*lane 6*); the *white arrow* indicates the 2 kb amplified in its linear form; total linear DNA of the non-lysogen *Anabaena* 77S15 digested by *Sau* 3A (*lane 7*), *Hind*III (*lane 8*) and undigested (*lane 9*); N(S)1 phage DNA (undigested) (*lane 10*). Molecular weights are given in kilobases. **b** The corresponding autoradiogram obtained after hybridization with the labelled 2 kb plasmid from the lysogen 84LS2 as a probe. The filter was exposed 2 h at room temperature for the *lanes 1* to *6*; it was exposed for 7 days at −80° C for *lanes 7* to *10*. Molecular weights are indicated in kilobases

tions were made: there is no homology between the 2 kb plasmid and DNA of the cyanophage N(S)1 (Fig. 4b, lane 10). On the contrary, both digested and non-digested DNA of *Anabaena* 77S15 showed significant hybridization with the plasmid of 84LS2 (Fig. 4b, lanes 7–9). Hybridization experiments with the labelled plasmid of 84LS2 to DNA of the 2 kb plasmid of 84LS1 (Fig. 4b, lanes 1, 2) and to total DNA of this latter lysogen (Fig. 4b, lanes 3–6) revealed the same pattern of labelling as that observed for total DNA of the non-lysogenic strain 77S15 (Fig. 4b, lanes 7–9) except that the hybridization signals were much stronger. These results show that the plasmids in the two lysogens are homologous and that this small plasmid is also present in the non-lysogenic *Anabaena* 77S15, but only at a much lower copy number. Thus, the integration of the prophage of N(S)1 into the chromosomes of the lysogens seems to lead to an important amplification of the 2 kb plasmid.

Discussion

Filamentous nitrogen-fixing cyanobacteria are abundant in tropical regions where they play an important role in the maintenance of the fertility of soils (Durrel 1964). Cyanophages that attack these organisms do not seem to be widely distributed in these habitats, since only one phage was isolated from fifty water samples collected at different sites in Senegal.

Based on its structural properties, a hexagonal capsid and a short tail, the cyanophage isolated from Senegal called N(S)1 has been assigned to the genus cyanopodovirus. Phages of similar size and morphology have been described to infect heterocystous cyanobacteria of the genus *Anabaena* and *Nostoc* (Khudyakov and Gromov 1973; Hu et al. 1981; Gromov 1983) or the filamentous non-heterocystous cyanobacteria of the LPP-group (Luftig and Haselkorn 1967).

The short tail of N(S)1 is rarely observed in uranyl acetate stained preparations and, as first suggested by Hu et al. (1981) for phages of this type, it is possible that the tail morphology changes during injection of phage DNA into the host in such a way that it emerges from the capsid. Consequently, the tails are only detectable in the post infection state of the phages.

As observed for other cyanophages (Beown 1972; Padan and Shilo 1973; Safferman 1973; Gromov 1983), N(S)1 has a narrow host range: only one *Anabaena* (77S15) out of 37 heterocystous cyanobacteria examined proved to be susceptible to lysis by this phage. All the cyanophages so far described for heterocystous cyanobacteria are capable of lysing both *Anabaena* PCC7120 or *Anabaena* variabilis PCC7118. Since N(S)1 does not attack *Anabaena* PCC7120, it seems to be, based on its host specificity, a new cyanophage. (N(S)1 exhibits a temperature range of stability greater than that reported for other cyanophages: 4° to 65° C rather than 4° to 50° C (Brown 1972; Padan and Shilo 1973; Safferman 1973) which could possibly be correlated with its tropical origin.

N(S)1 did not show any immunological relatedness to the cyanophage N1. The immunological relationship of N(S)1 to other members of the same genus cyanopodovirus, needs to be examined.

N(S)1 produces turbid plaques indicative of a temperate phage rather than of a virulant phage which typically produces clear plaques. We therefore attempted to isolate lysogens for N(S)1, and two stable lysogenic clones, 84SL1 and 84SL2, were obtained. The development of the prophage can be induced with mitomycin C as has previously been reported for the prophages of LPP-1D and LPP-2 of lysogenic strains of *Plectonema boryanum* (Cannon et al. 1971; Cannon and Shane 1972) and of N-1 in a lysogen of *Nostoc muscorum* ISU (*Anabaena* PCC7120) (Padhy and Singh 1978). Since no extrachromosomal DNA of a molecular weight equivalent to that of the cyanophage N(S)1 (i.e 28 kb) was detected in the lysogenic strains of *Anabaena* 77S15, we conclude that the prophage is likely to be integrated into the chromosome. Hybridization experiments with purified phage DNA as a probe are to be performed to confirm this result.

The significance and the mechanisms of the amplification of a small 2 kb plasmid in the lysogens that is present only at a low copy number in the non-lysogenic strain of *Anabaena* 77S15 cannot be explained at present, but should be examined further, particularly, since this is the first report of such a change in plasmid content in a lysogenic cyanobacterium.

Acknowledgements. We are very grateful to Prof. G. Stanier and to Dr. R. Rippka for numerous and helpful advice, for critical reading of the manuscript and for gift of cyanobacterial strains and cyanophages. We also thank Dr. X. Mattei (Faculté des Sciences de Dakar) for electron microscopy. DNA purification and hybridization experiments were performed in the Unité de Physiologie Microbienne, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, Paris, France.

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Received January 27, 1987/Accepted May 6, 1987