Gene, 20 (1982) 103–110
Elsevier Biomedical Press

Nostoc PCC7524, a cyanobacterium which contains five sequence-specific deoxyribonucleases

(Gene transfer; restriction-modification; cleavage sites)

John Reaston, Marja G.C. Duyvesteyn *, and Adrian de Waard *

*Department of Molecular Cell Biology, State University of Utrecht, Padualaan 8, 3508 TB Utrecht, and * Department of Medical Biochemistry, Sylvius Laboratories, State University of Leiden, Wassenaarseweg 72, 2333 AL Leiden (The Netherlands)*

(Received April 26th, 1982)
(Accepted July 26th, 1982)

SUMMARY

Five nucleotide sequence-specific deoxyribonucleases present in cell-free extracts of the filamentous cyanobacterium *Nostoc* PCC7524 have been purified and characterized. One of these enzymes, designated *Nsp*(7524)I cleaves at a new kind of nucleotide sequence, i.e. 5′–PuCATG↓Py–3′. The other four restriction enzymes in this organism, designated *Nsp*(7524)II, *Nsp*(7524)III, *Nsp*(7524)IV and *Nsp*(7524)V, are isoschizomers of enzymes which have been previously described. The cleavage site of *Nsp*(7524)II which is an isoschizomer of *Sdu*I was determined.

INTRODUCTION

Nostoc PCC7524 is a photosynthetic prokaryote which was isolated from a hot spring in Sri Lanka and subsequently purified into axenic culture at the Institut Pasteur, Paris (Rippka et al., 1979). It was placed into the section IV of cyanobacteria under the classification system suggested by Rippka et al. (1979). This group is composed of filamentous cyanobacteria which are collectively distinguishable from the section III organisms by their capacity for cellular differentiation. They differ from the section V cyanobacteria in the mode of their cell division and akinete germination. *Nostoc* PCC7524 possesses several properties of great interest for genetic study. It performs oxygenic "plant-like" photosynthesis, fixes N_2 aerobically and exhibits cellular differentiation i.e. the formation of akinetes, heterocysts, hormogonia and vegetative cells (Sutherland et al., 1979).

The development of a self-cloning system in *Nostoc* PCC7524 and other filamentous cyanobacteria would greatly improve our knowledge and understanding of photosynthesis, nitrogen fixation and cell differentiation in this important group of micro-organisms. However, no conclusive gene-transfer system has yet been demonstrated in

Abbreviations: *Asu*, endoR nuclease from *Anabaena subcylindrica*: *Ava*, endoR nuclease from *Anabaena variabilis*; DEAE, diethylaminoethyl; *Nsp*, *Nostoc* species; PCC, Pasteur Culture Collection; Z, restriction fragments of bacteriophage ϕX174 RF DNA produced by endonuclease R *Hae*III (Sanger et al., 1978).

filamentous cyanobacteria (Doolittle, 1979) similar to that described for the unicellular organisms *Anacystis nidulans* R2 (Shestakov and Khyen, 1970; Van den Hondel et al., 1980), *Aphanocapsa* PCC6714 (Astier and Joset-Espardellier, 1976), *Gloeocapsa alpicola* (Devilly and Houghton, 1977) and *Agmenellum quadruplicatum* (Stevens and Porter, 1980). Thus, the genetics of filamentous cyanobacteria is a long way behind what is known for other bacteria for which a gene-transfer system is available.

Attempts to transform *Nostoc* PCC7524 with chromosomal and plasmid DNA or to obtain conjugation using promiscuous plasmid DNA have been unsuccessful to date (unpublished observations). This apparent lack of gene transfer may possibly be due to the breakdown of the donor DNA by deoxyribonuclease activity or possibly lack of uptake of the DNA, but this remains to be elucidated. In a recent study, Reaston et al. (1980; 1982) reported that plasmid pDU1 from *Nostoc* PCC7524 was not cleaved by a large number of restriction enzymes which they tested. This may either be because the plasmid lacks the recognition sequences for the restriction enzymes tested or because *Nostoc* PCC7524 contains a restriction-modification system which prevents the ready digestion of its DNA. The above findings stimulated us to investigate whether *Nostoc* PCC7524 contains sequence-specific endonucleases which may explain the lack of gene transfer and the paucity of restriction sites in this organism. In this paper we report on the isolation and characterization of five type-II restriction endonucleases from *Nostoc* PCC7524.

MATERIALS AND METHODS

(a) Organism and growth conditions

Nostoc PCC7524 was obtained from the Pasteur Culture Collection, Paris (Rippka et al., 1979). It is also deposited in the American Type Culture Collection (ATCC29411). The growth medium and conditions used were as before (Sutherland et al., 1979). Checks on bacterial contamination were carried out routinely (Van den Hondel et al., 1979) and only axenic cultures were used.

(b) Chemicals

[γ-^{32}P]ATP (spec. act. 2000–3000 Ci/mmol) was purchased from The Radiochemical Centre (Amersham, U.K.) or from New England Nuclear. Acrylamide (99%) and dimethyl sulfate (98%) were from Merck-Schuchardt (Munich); bis-acrylamide (ultra pure) and hydrazine (95%) were from Eastman Kodak Co. (Rochester, NY); urea (99.5%) was purchased from Bethesda Research Laboratories (Rockville, MD); piperidine (98%) was from Baker Chemicals (Deventer). All other chemicals were of the highest quality available.

(c) Purification of enzymes

Frozen packed cells (10–30 g) were processed according to Duyvesteyn and De Waard (1980). The crude enzyme preparation was passed through a 20 × 2.5 cm column of phosphocellulose (Whatman P11) equilibrated with 20 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 0.2 mM $MgCl_2$, 2 mM mercaptoethanol and eluted with a linear gradient of 0–0.6 M NaCl in the same buffer also containing 10% glycerol. Enzyme fractions were monitored with λ DNA as substrate and by gel electrophoretic examination (Sugden et al., 1975). Subsequent purification was on columns of DEAE cellulose (Whatman DE52) or pyran-Sepharose (Chirikjian et al., 1975).

(d) Determination of cleavage specificity

This was done using methods described before (Duyvesteyn and De Waard, 1980; Maxam and Gilbert, 1980; Tu and Wu, 1980). In the case of suspected isoschizomers cleavage patterns on agarose gel were compared.

(e) Nomenclature of sequence-specific endonucleases

The proposals of Smith and Nathans (1973) were followed except that the species symbols were replaced by the letters *sp* followed by the strain number. This was necessary because strains in the

Pasteur Culture Collection were not given species names by Rippka et al. (1979). Thus, the endonucleases studied in this paper are designated *Nsp*(7524)I, *Nsp*(7524)II, etc. Roberts (1982) designated one of the enzymes from *Nostoc* PCC7524 as *Nsp*CI which is identical to *Nsp*(7524)I. In the present paper the strain number 7524 is used throughout.

RESULTS

(a) Isolation of sequence-specific endonucleases

Chromatography on phosphocellulose of cell-free extracts from *Nostoc* PCC7524 revealed three major peaks of enzyme activity, which eluted at approx. 0.1, 0.25 and 0.35 M NaCl, respectively. Subsequent rechromatography of the middle peak activity on either DEAE cellulose or pyran-Sepharose revealed that these fractions contained three major sequence-specific endonucleases plus some minor activity of the two endonucleases which eluted early and late from the phosphocellulose column. In the following we describe the characterization of the sequence-specific endonucleases in the order *Nsp*(7524)I to *Nsp*(7524)V. Subsequent steps in their purification are given under the appropriate headings.

(b) Characterization of *Nsp*(7524)I

This enzyme eluted last from the phosphocellulose column at 0.35 M NaCl. It was abundant and of sufficient purity to allow characterization of its cleaving properties towards the DNA substrates plasmid pAT153 (Twigg and Sheratt, 1980) and bacteriophage M13 RF DNA (Van Wezenbeek et al., 1980) in two ways. First pAT153 was cleaved with *Sal*I, then 5′-end labeled with ^{32}P by T4 polynucleotide kinase. It was cleaved further with *Bam*HI and separated into two fragments by polyacrylamide slab gel electrophoresis. The fragment between the *Sal*I and *Bam*HI sites (275 bp long), known to contain a cleavage site for *Nsp*(7524)I was eluted from the gel. A 5% aliquot of this was incubated with this enzyme and run on a polyacrylamide slab gel in a fifth lane next to a sequence ladder prepared from the remaining 95% of the fragment. Fig. 1 shows an autoradiograph indicating that the cleavage site for *Nsp*(7524)I resides in the sequence 5′–GCATGC–3′, probably between the rightmost G and C.

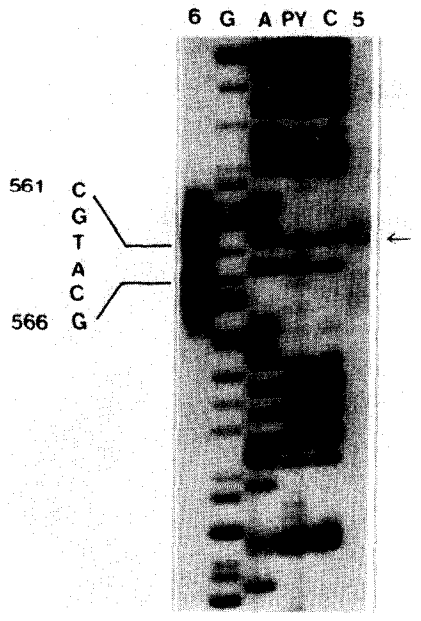

Fig. 1. Determination of specific cleavage site for *Nsp*(7524)I in plasmid pAT153. Inner four lanes indicate nucleotide sequence as determined according to Maxam and Gilbert. Recognition sequence is GCATGC. Site of cleavage (between G and C at the right) follows from position of *Nsp*(7524)I-fragment in lanes 5 and 6. The former was obtained using a shorter exposure time. Note that the structure of the enzymatic product (lanes 5 and 6) is of the nature 5′–pGCATG-OH′–3′, which migrates slightly slower than the chemically degraded product of the same sequence 5′–pGCATGp–3′ carrying a 3′-terminal phosphate.

In the second approach, bacteriophage M13 RF DNA was cleaved with *Nsp*(7524)I yielding five fragments, which were then ^{32}P-labeled at their 5′-ends. After gel electrophoresis and elution two of these were separated into single strands, degraded with pancreatic DNase and analyzed by the wandering spot method of Tu and Wu (1980). Fig. 2 shows the results for one of these fragments. Its 5′-terminal pT forms part of the hexanucleotide sequence 5′–ACATGT–3′. To check whether the asymmetrical hexanucleotide sequence

5′–ACATGC–3′
3′–TGTACG–5′

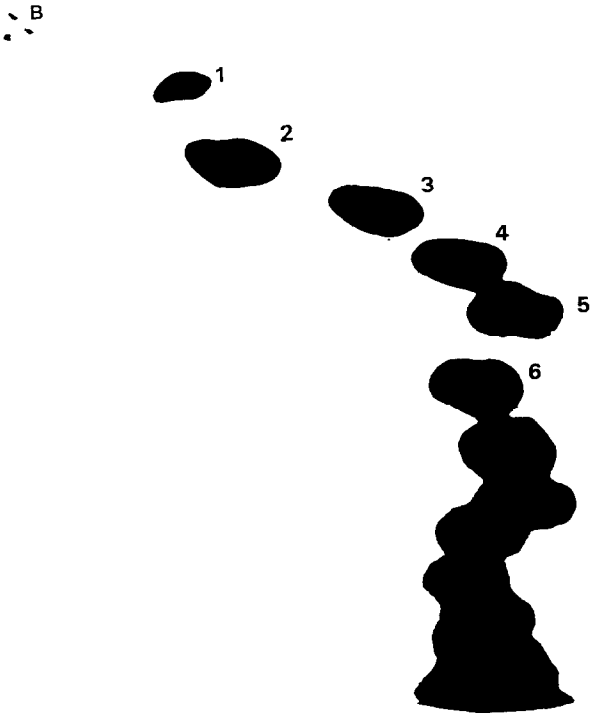

Fig. 2. Wandering spot analysis of M13 DNA oligonucleotides terminating in cleavage site for *Nsp*(7524)I (coordinates: 3722–3717, lower strand). Nucleotide 3717 was shown to be pT by snake venom exonuclease digestion and electrophoretic examination. Spot 1, pTpA (coordinates 3717–3716); spot 2, pTpApA; spot 3, pTpApApT; spots 4 and 5 have additional T residues, whereas spot 6 has an additional A. Published sequence (Van Wezenbeek et al., 1980) reads from coordinate 3722–3711: 5′ACATGTAATTTA. B, blue marker.

is also cleaved by *Nsp*(7524)I a computer search was made for it in DNAs of known nucleotide sequence. A *Hin*dIII fragment of adenovirus 5 DNA (Ad5 *Hin*dIII-C, running from 17–31% genome length) contains this asymmetric sequence three times (B.M.M. Dekker and H. van Ormondt, personal communication). Sequence analysis of this fragment revealed that these asymmetric sites are indeed cut by this enzyme. Thus, *Nsp*(7524)I recognizes and cleaves the sequence 5′–PuCATG$^{\downarrow}$Py–3′ as shown.

(c) Characterization of *Nsp*(7524)II

Fractions which eluted from the phosphocellulose column at approx. 0.25 M NaCl containing this enzyme were heavily contaminated with other endonucleases. It was freed from most of these other activities by chromatography on pyran-Sepharose, eluting at approx. 0.3 M KCl. A digest of bacteriophage φX174 DNA with *Hae*III was shown to contain cleavage sites for *Nsp*(7524)II in three fragments designated Z6b, Z7 and Z9 (Sanger et al., 1978). The latter two fragments after ^{32}P labeling at their 5′-ends were cleaved with *Nsp*(7524)II and analyzed as shown in Fig. 3A and B, respectively. Fragment Z7 contained the sequence GTGCT$^{\downarrow}$ adjacent to the point of *Nsp*(7524)II cleavage (coordinates 538–542, Sanger et al., 1978), while Z9 contained the sequence GTGCA$^{\downarrow}$ at the 3′-side of the site of cleavage (coordinates 4784–4780). In both cases the nucleotide to the right of the arrow is a cytosine residue. The provisional conclusion was that *Nsp*(7524)II recognizes and cleaves the sequence with the general structure 5′–G$^{A}_{T}$GC$^{T}_{A}$C–3′. Further investigations with an *Nsp*(7524)II digest of SV40 DNA revealed that the recognition sequence is of a more degenerate nature.

We have analyzed digests of SV40 DNA with purified *Nsp*(7524)II and shown by sequence analysis that this enzyme cleaves the following sequence: GGGCTC (coordinates 776–781, Buchman et al., 1981), GGGCAC (1288–1293), GGGCCC (2258–2263), and GTGCCC (3385–3390). Thus, the sequences recognized by *Nsp*(7524)II belong to the general structure:

$$\begin{array}{l} \quad\ G\quad C \\ 5'\text{–}G\,A\,G\,C\,T\,C\text{–}3' \\ \quad\ T\quad A \end{array}$$

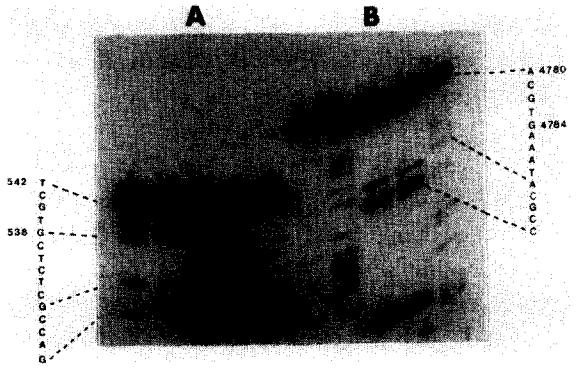

Fig. 3. Sequence of 3′ ends at the *Nsp*(7524)I cut. (A) Sequence gel showing GTGCT-OH at the 3′-end of φX174 RF DNA Z7 fragment (upper strand) cut with *Nsp*(7524)II. (B) Sequence gel showing GTGCA-OH at the 3′-end of Z9 fragment (lower strand) cut with *Nsp*(7524)II.

Thus *Nsp*(7524)II is an isoschizomer of *Sdu*I from *Streptococcus durans* which has been reported recently by Janulaitis et al. (1981). These authors did not determine the precise cleavage site of *Sdu*I. We determined the cleavage site of *Nsp*(7524)II to be as shown above by a "fifth-lane" experiment of the SV40 DNA site at coordinates 776–781. Fig. 4 shows this to be as postulated.

We were also able to exclude the possibility that the second nucleotide of the recognized hexanucleotide can be cytosine which would turn the sequence into 5′–GNGCNC–3′. The latter structure occurs 8 times in the bacteriophage ϕX174 genome. However, extensive cleavage of ϕX174 RF DNA with purified *Nsp*(7524)II gave only three fragments. Their size was estimated to be 3752, 1145 and 489 bp which would agree with cleavage sites at locations 538, 4779 and 4290 which have the sequence GTGCTC, GTGCAC and GTGCTC, respectively. The former two cases are documented in Fig. 3A and B.

(d) Characterization of *Nsp*(7524)III

This enzyme eluted also at approx. 0.25 M NaCl from phosphocellulose. It was purified by gradient elution from DEAE cellulose, appearing at 0.20 M KCl. Its cleavage site was determined to be identical to that of *Ava*I (Hughes and Murray, 1980): 5′–C↓PyCGPuG–3′. This factor was established by applying both types of sequencing strategies (described in METHODS) to digests of ϕX174 RF DNA. Fig. 5 shows an example of a wandering

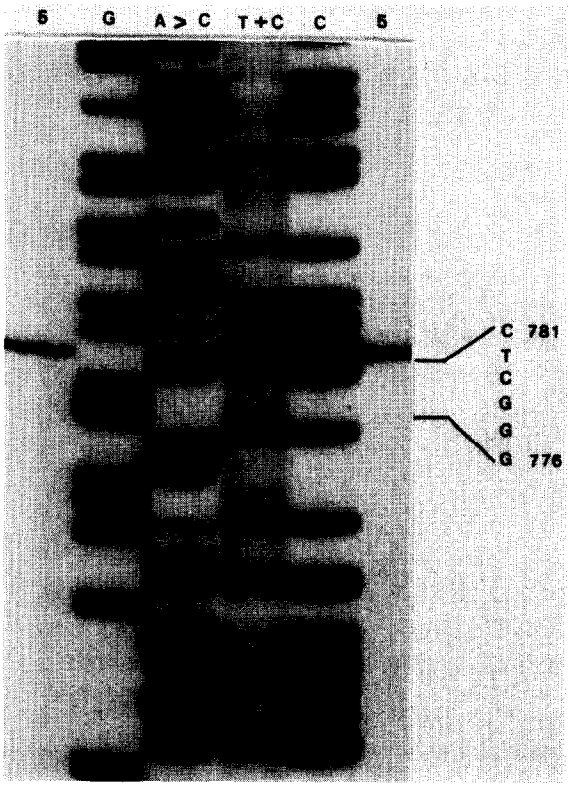

Fig. 4. Nucleotide sequence in *Ava*II-E fragment of SV40 DNA (coordinates 589–1021) at the cleavage site of *Nsp*(7524)II (coordinates 776–781). Note that the structure of the enzymatic product ("lane 5", applied at both sides of the ladder) is of the nature 5′–pGGGCT-OH′–3′ which migrates slightly slower than the chemically degraded product of the same sequence 5′–pGGGCTp–3′ carrying a 3′-terminal phosphate. Cleavage is made in sequence GGGCT↓C as shown. The T+C lane is rather weak.

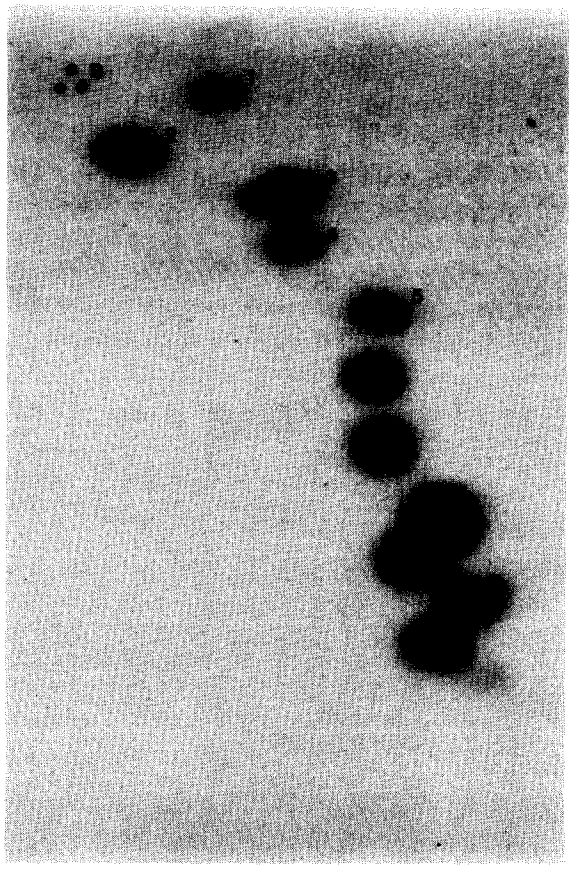

Fig. 5. Wandering spot analysis of ϕX174 oligonucleotides terminating in single cleavage site 5′–C↓TCGAG–3′ (coordinates 162–167) for *Nsp*(7524)III. Spot 1, pT (checked electrophoretically); spot 2, pTpC; spot 3, pTpCpG; spot 4, pTpCpGpA; spot 5, pTpCpGpApG. B, blue marker.

spot analysis of the cleavage site between nucleotides 162–163 (Sanger et al., 1978). The cleavage pattern of *Nsp*(7524)III as examined by agarose gel electrophoresis was identical to that of *Ava*I.

(e) Characterization of *Nsp*(7524)IV

This enzyme was also heavily contaminated with other nucleolytic activities as it was eluted from the phosphocellulose column at 0.25 M NaCl. It was purified on DEAE cellulose (applying a gradient from 0–0.25 M KCl) and shown to be an isoschizomer of *Asu*I (Hughes et al., 1980), by an examination of its cleavage pattern on agarose gel electrophoresis. *Nsp*(7524)IV recognizes specifically the nucleotide sequence G ↓ GNCC. This was established by analysis of digests of SV40 DNA (see Buchman et al., 1981) and φX174 RF DNA (Sanger et al., 1978) obtained after incubation of these substrates with purified *Nsp*(7524)IV. One example out of many is shown in Fig. 6. The site of cleavage was determined to be between the two guanine residues by means of the wandering spot method. (The latter technique is superior to the Maxam and Gilbert sequencing method for this limited purpose.) This was conclusively shown with the help of snake venom exonuclease and paper electrophoresis.

(f) Characterization of *Nsp*(7524)V

This enzyme eluting at 0.1 M NaCl from phosphocellulose was sufficiently pure to have its specificity determined directly. Its recognition sequence is apparently identical to that of *Asu*II: TTCGAA (De Waard and Duyvesteyn, 1980), as shown in Fig. 7.

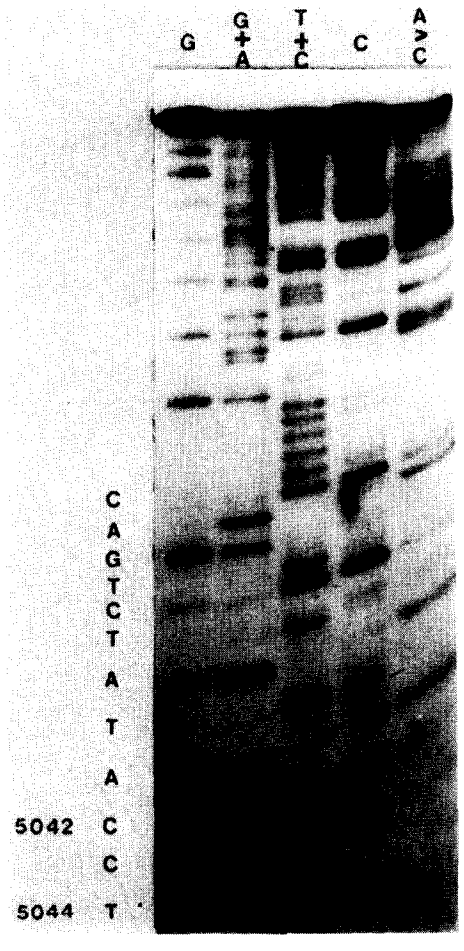

Fig. 6. Polyacrylamide gel (20%) showing φX174 nucleotide sequence near cleavage site of *Nsp*(7524)IV. Coordinates of recognition site GGTCC on lower strand are 5046–5042. The 5′-terminal nucleotide was shown to be pG (coordinate 5045), not pT(5044) by digestion with snake venom exonuclease.

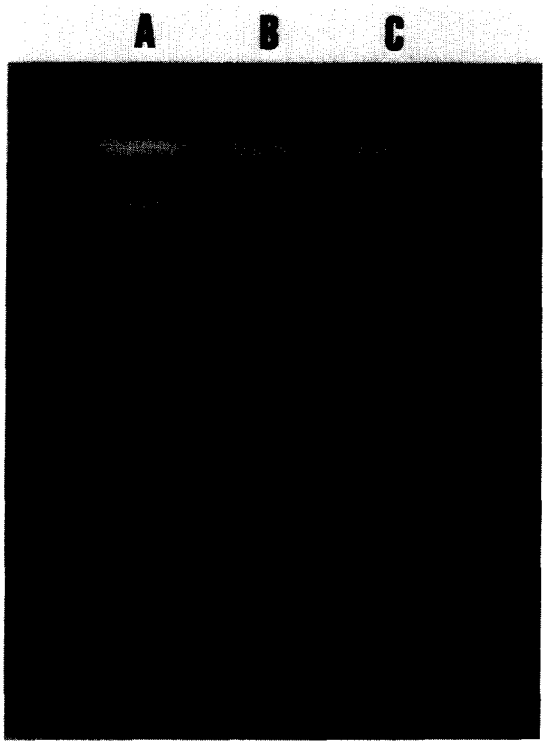

Fig. 7. Comparison of cleavage patterns on agarose gel. A, *Asu*II + λDNA; B, *Nsp*(7524)V + λDNA; C, *Asu*II + *Nsp*(7524)V + λDNA.

DISCUSSION

In this paper we have reported the presence of five sequence-specific endonucleases in extracts of one cyanobacterial strain. One of these, *Nsp*(7524)I recognizes a new nucleotide sequence, PuCATG$^{\downarrow}$Py. (Note that a subset of this hexamer, GCATG$^{\downarrow}$C, is the recognition site for *Sph*I from *Streptomyces phaeochromogenes*; Fuchs et al., 1980.) Three of the endonucleases are isoschizomers of enzymes which have been encountered before in other cyanobacteria: *Ava*I (C$^{\downarrow}$PyCGPuG), *Asu*I (G$^{\downarrow}$GNCC) and *Asu*II (TT$^{\downarrow}$CGAA), while one other resembles a bacterial endonuclease, *Sdu*I (G$\begin{matrix}\mathrm{A}\\\mathrm{T}\end{matrix}GC\begin{matrix}\mathrm{T}\\\mathrm{A}\end{matrix}C^{\downarrow}$C).

The possibility that we have been dealing with contaminated cultures of *Nostoc* PCC7524 is very remote as stringent checks on bacterial contamination were made. In addition we have performed four independent enzyme isolations from cultures grown from pure inocula in different locations. One batch was obtained as a frozen pellet from Dr. C.P. Wolk (Michigan State University). In all experiments the same results were obtained.

Little is known about the in vivo function(s) of sequence-specific endonucleases in cyanobacteria. The paucity of restriction sites in plasmid pDU1 from *Nostoc* PCC7524 (Reaston et al., 1982) may be due to its extensive modification possibly as a result of the presence of five R-M systems. Reliable gene transfer may also be prevented in *Nostoc* PCC7524 because of the presence of no less than five restriction endonucleases. Future investigators wishing to develop gene-transfer systems in filamentous cyanobacteria may therefore be advised to work with strains known to be deficient in such enzymes.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. H. van Ormondt for his advice and for providing computer-stored information on nucleotide sequences. Dr. C.P. Wolk of Michigan State University kindly supplied a frozen pellet of *Nostoc* PCC7524. Dr. P.D. Baas (Utrecht) kindly supplied ϕX174 RF DNA. Professor G.A. van Arkel extended to one of us (J.R.) hospitality in his laboratory.

This work was supported in part by the Foundation of Chemical Research in The Netherlands (SON). The receipt of a European Science Exchange Programme Fellowship from the Royal Society to J.R. is gratefully acknowledged.

REFERENCES

Astier, C. and Joset-Espardellier, F.: Mise en évidence d'un système de transfert génétique chez une cyanophycée du genre *Aphanocapsa*. C.R. Acad. Sci. Paris 282 (1976) 795–797.

Buchman, A.R., Burnett, L. and Berg, P.: The SV40 nucleotide sequence, in Tooze, J. (Ed.), Molecular Biology of Tumor Viruses, 2nd edn: DNA Tumor Viruses (revised). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1981, pp. 799–841.

Chirikjian, J.G., Rye, L. and Papas, T.S.: Affinity chromatography of viral DNA polymerases on pyran-Sepharose. Proc. Natl. Acad. Sci. USA 72 (1975) 1142–1146.

Devilly, C.I. and Houghton, J.A.: A study of genetic transformation in *Gloeocapsa alpicola*. J. Gen. Microbiol. 98 (1972) 277–280.

De Waard, A. and Duyvesteyn, M.: Are sequence-specific deoxyribonucleases of value as taxonomic markers of cyanobacterial species? Arch. Microbiol. 128 (1980) 242–247.

Doolittle, W.F.: The cyanobacterial genome, its expression and the control of that expression. Adv. Microb. Physiol. 20 (1979) 1–102.

Duyvesteyn, M. and De Waard, A.: A new sequence-specific endonuclease from a thermophilic cyanobacterium, *Mastigocladus laminosus*. FEBS Lett. 111 (1980) 423–426.

Fuchs, L.Y., Covarrubias, L., Escalante, L., Sanchez, S. and Bolivar, F.: Characterization of a site-specific restriction endonuclease *Sph*I from *Streptomyces phaeochromogenes*. Gene 10 (1980) 39–46.

Hughes, S.G. and Murray, K.: The nucleotide sequences recognized by endonucleases *Ava*I and *Ava*II from *Anabaena variabilis*. Biochem. J. 185 (1980) 65–75.

Hughes, S.G., Bruce, T. and Murray, K.: The isolation and characterization of a sequence-specific endonuclease from *Anabaena subcylindrica*. Biochem. J. 185 (1980) 59–63.

Junalaitis, A., Marcinkevičienė, L., Petrušytė, M. and Mironov, A.: A new sequence-specific endonuclease from *Streptococcus durans*. FEBS Lett. 134 (1981) 172–174.

Maxam, A.M. and Gilbert, W.: Sequencing end-labeled DNA with base-specific cleavages, in Grossman, L. and Moldave, K. (Eds.), Methods in Enzymology, Vol. 65. Academic Press, New York, 1980, pp. 499–560.

Reaston, J., Van den Hondel, C.A.M.J.J., Van der Ende, A., Van Arkel, G.A., Stewart, W.D.P. and Herdman, M.: Com-

parison of plasmids from the cyanobacterium *Nostoc* PCC7524 with two mutant strains unable to form heterocysts. FEMS Microbiol. Lett. 9 (1980) 185–188.

Reaston, J., Van den Hondel, C.A.M.J.J., Van Arkel, G.A. and Stewart, W.D.P.: A physical map of plasmid pDUI from the cyanobacterium *Nostoc* PCC7524. Plasmid 7 (1982) 101–104.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y.: Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111 (1979) 1–61.

Roberts, R.J.: Restriction and modification enzymes and their recognition sequences. Nucl. Acids Res. 10 (1982) 117–144.

Sanger, F., Coulson, A.R., Friedman, T., Air, G.M., Barrell, B.G., Brown, N., Fiddes, J.C., Hutchison III, C.A., Slocombe, P.M. and Smith, M.: The nucleotide sequence of bacteriophage ϕX174. J. Mol. Biol. 125 (1978) 225–246.

Shestakov, S.V. and Khyen, N.T.: Evidence for genetic transformation in the blue green alga *Anacystis nidulans*. Mol. Gen. Genet. 107 (1970) 372–375.

Smith, H.O. and Nathans, D.: A suggested nomenclature for bacterial host modification and restriction systems and their enzymes. J. Mol. Biol. 81 (1973) 419–423.

Stevens, S.E. and Porter, R.D.: Transformation in *Agmenellum quadruplicatum*. Proc. Natl. Acad. Sci. USA 77 (1980) 6052–6056.

Sugden, B., De Troy, B., Roberts, R.J. and Sambrook, J.: Agarose slab gel electrophoresis equipment. Anal. Biochem. 68 (1975) 36–46.

Sutherland, J.M., Herdman, M. and Stewart, W.D.P.: Akinetes of the cyanobacterium *Nostoc* PCC7524: macromolecular composition, structure and control of differentiation. J. Gen. Microbiol. 115 (1979) 273–287.

Tu, C.D. and Wu, R.: Sequence analysis of short DNA fragments, in Grossman, L. and Moldave, K. (Eds.), Methods in Enzymology, Vol. 65. Academic Press, New York, 1980, pp. 620–638.

Twigg, A.J. and Sheratt, D.: Trans-complementable copy number mutants of plasmid ColE1. Nature 283 (1980) 216–218. (For a correction of this paper see Old, R.W. and Primrose, S.B. in Principles of Gene Manipulation (2nd ed.), Blackwell, Oxford, 1981, Fig. 3.16, p. 47.)

Van den Hondel, C.A.M.J.J., Keegstra, W., Borrias, W.E. and Van Arkel, G.A.: Homology of plasmids of unicellular cyanobacteria. Plasmid 2 (1979) 323–333.

Van den Hondel, C.A.M.J.J., Verbeek, S., Van der Ende, A., Weisbeek, P.J., Borrias, W.E. and Van Arkel, G.A.: Introduction of transposon Tn*901* into a plasmid of *Anacystis nidulans*: preparation for cloning in cyanobacteria. Proc. Natl. Acad. Sci. USA 77 (1980) 1570–1574.

Van Wezenbeek, P.M.G.F., Hulsebos, Th.J.M. and Schoenmakers, J.J.G.: Nucleotide sequence of the filamentous bacteriophage M13 DNA genome: comparison with phage fd. Gene 11 (1980) 129–148.

Communicated by H.O. Smith.