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Sequence-Specific Endonucleases in Strains of *Anabaena* and *Nostoc*

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Abstract. The complements of restriction endonucleases of 12 strains of cyanobacteria were determined in cell-free extracts, and were compared with the complements of restriction activities assessed by measuring the relative efficiencies of plating of cyanophages on those cyanobacteria. The hosts which were susceptible to all of the phages contained endo R · *Ava*I and endo R · *Ava*II, and in several cases probably endo R · *Ava*III, or isoschizomers of these enzymes. Three hosts which were lysed by only a subset (1 or 3) of the phages contained different restriction endonucleases. *Anabaena* sp. PCC 7120 showed apparent phenotypic restriction of phage AN-22 grown in hosts with (isoschizomers of) *Ava*I, II and III, but no corresponding endonuclease has yet been detected in vitro. Nostoc sp. ATCC 29131 (PCC 6705) was found to contain a restriction enzyme, *Nsp*BII, with hitherto unknown specificity, $C\left(\frac{A}{C}\right)GC\left(\frac{T}{G}\right)G$.

Key words: Restriction endonucleases – Cyanophages – DNA recognition

The occurrence of sequence-specific restriction endonucleases can be a major impediment to interspecific and intergeneric transfer of DNA (Arber and Linn 1969). We are interested in developing systems for such transfer in heterocyst-forming cyanobacteria because transmission genetics using DNA cloned in other hosts would facilitate the analysis, in the cyanobacteria, of oxygenic photosynthesis, protection of nitrogenase from oxygen, and differentiation and pattern formation. The relative plating efficiencies of cyanophage N-1, grown on two of its hosts, *Anabaena variabilis* 29413 and *Anabaena* sp. 27893 (PCC 7120), and plated on those hosts, provided support for the view that the former organism contains a restriction endonuclease which is absent from the latter (Currier and Wolk 1979). In fact, direct analysis showed the presence in only the former, of endo R · *Avr*II, specific for CCTAGG (Rosenvold and Szybalski, cited in Roberts 1983; see Table 1). Subsequently, numerous cyanophages were isolated which are capable of lysing diverse strains of heterocyst-forming cyanobacteria of the genera *Anabaena* and *Nostoc* (Hu et al. 1981). These cyanophages provided an opportunity to determine whether a correlation could be found between apparent restriction, as suggested by plating efficiencies, and sequence-specific restriction endonucleases, as identified in cell-free extracts of the different strains. Had it been found that one or another of the cyanobacterial strains lacked restriction systems, that strain would have seemed an organism of choice to serve as a recipient for recombinant DNA. Conversely, restriction of cyanophage DNA coming from a source with known restriction specificities can point to additional, and perhaps novel, restriction specificities in the restricting cyanobacterium.

Materials and Methods

The strains which we have studied are listed in Table 1. The catalog number in both the American Type Culture Collection (ATCC) and the Pasteur Culture Collection (PCC) are given. For the histories of some of these strains the reader is referred to Rippka et al. (1979). Cyanobacteria were grown at 23° C or 30° C in 16- or 40-l batch cultures in an eight-fold

Table 1. Cyanobacterial strains analyzed

Organism (source)	ATCC	PCC
Anabaena species	27893	7120
Anabaena species	29151	7119
Anabaena species	27898	6411
Anabaena variabilis (Myers strain, U. of Leningrad 458)	probably derived from	
Anabaena variabilis M-3 (University of Tokyo)	27892	7118
Anabaena variabilis M-2 (same)	possibly derived from 27892	7118
Nostoc muscorum M-131 (same) from Gerloff		
Anabaena variabilis	29413[a]	7937
Nostoc linckia (from the late R. N. Singh)		
Nostoc species	29105	6719
Nostoc species	29106	7413
Nostoc species	29131	6705

[a] This organism is called *Anabaena variabilis* UW in the list of restriction endonucleases by R. J. Roberts (1983) and abbreviated there as *Avr* (E. C. Rosenvold, personal communication). It was derived from the Collection of the University of Texas and known as UTEX 1444; it had formerly been named *Anabaena flos-aquae* and is included under the name in the list of strains of the Culture Collection of Algae and Protozoa in Cambridge (UK), CCAP 1403/13a

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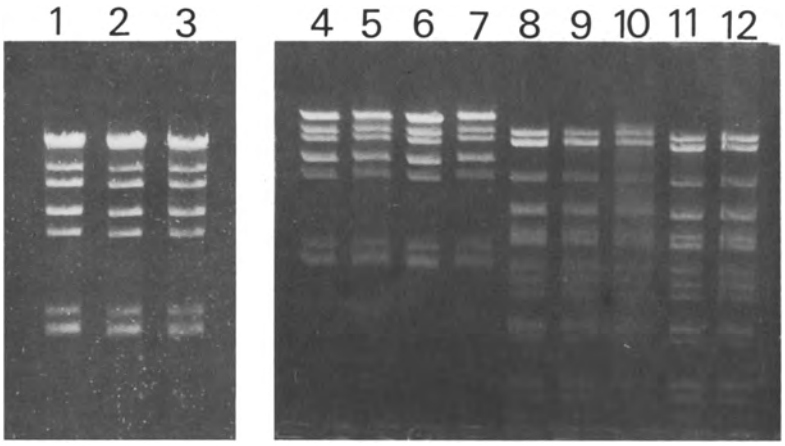

Fig. 1
Identity of cleavage patterns of bacteriophage λ DNA by isoschizomers of endo R · *Ava*I (1–7) and *Ava*II (8–12). *1 Ava*I from PCC 7120; *2 Ava*I from New England Biolabs; *3* Mixture of (*1*) and (*2*); *4 Avr*I from ATCC 29413; *5 Avr*I from CCAP 1403/13a; *6 Ava*I from PCC 7120; *7 Ava*I from Leningrad strain 458; *8 Fdi*I from *Fremyella diphosiphon* (PCC 7601) for which the recognition sequence GG$\left(\frac{A}{T}\right)$CC has been rigorously established (Van den Hondel et al. 1983); *9 Ava*II from PCC 6411; *10 Ava*II from PCC 7120; *11 Ava*II from *Nostoc linckia*; *12 Sin*I from *Salmonella infantis* (Lupker and Dekker 1981)

dilution of the medium of Allen and Arnon (1955). The medium was sterilized in a 500-l Nutrient Sterilizer (New Brunswick Scientific Corp. Edison, NJ, USA). For strains incapable of aerobic fixation of dinitrogen, the medium was supplemented with 5 mM $NaNO_3$ plus 5 mM KNO_3. The cultures were harvested by continuous flow centrifugation, tested for contamination, frozen on dry ice, and kept frozen until analyzed for restriction endonucleases.

The sequence-specific endodeoxyribonucleases were isolated essentially as described earlier (Duyvesteyn and de Waard 1980; de Waard and Duyvesteyn 1980). Pellets (approximately 7–10 g wet weight) were thawed, then frozen and thawed three times, and finally broken in a French pressure cell at 2,500 kg/cm^2. The paste was diluted in a 150 ml stainless steel beaker with 100 ml of buffer A (10 mM Tris · HCl, pH 7.4, 0.2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM mercaptoethanol) and sonicated at 0° C for five 1-min periods with cooling in ice-water. Cell debris was removed by centrifugation at 20,000 × g for 45 min. Nucleic acids were precipitated by addition of polyethylene imine (Polymin P, BASF, neutralized with HCl to pH 7.8) to a final concentration of 1 %. The precipitate was extracted three times with 1/5 volume of 0.6 M NaCl in buffer A (based on the volume of the original crude extract). Solid $(NH_4)_2SO_4$ was added to the supernatant of the polyethylene imine-step to 70 % saturation. The resulting precipitate was dissolved in a small volume of buffer B (20 mM potassium phosphate, pH 7.4, 0.2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM mercaptoethanol), combined with the 0.6 M NaCl-extract and dialyzed against 2 l of buffer B with one change of buffer. The cyanobacterial endonucleases were purified in either of two ways. In the first method the enzyme was chromatographed on phosphocellulose (Whatman P11) as described earlier (de Waard et al. 1978). In the second method the dialyzed crude enzyme was fractionated with ammonium sulphate as described by Murray et al. (1976). The two fractions obtained were each chromatographed on DEAE cellulose (Whatman DE52) using a salt gradient. The fractions from these columns were assayed by agarose gel electrophoretic examination of the banding patterns obtained by incubation with bacteriophage λ DNA. If necessary, the enzymatically active fractions were purified further by chromatography on DEAE cellulose, phosphocellulose or heparin-Sepharose (de Waard et al. 1979). If the banding patterns of digests of λ DNA could not be identified by their resemblance to known patterns of various cyanobacterial endonucleases, smaller DNA molecules of known sequence: SV40 DNA (Buchman et al. 1981), ØX174 RF DNA (Sanger et al. 1978), pBR322 (Sutcliffe, 1978) or fragments of adenovirus 5 DNA cloned in plasmid pAT153 (Bernards et al. 1982) were incubated with the enzyme. The nature of the cleavage sites was identified with the help of sequencing procedures (Maxam and Gilbert 1980; Tu and Wu 1980).

Phages specified as having been grown in any particular host were cloned three successive times in that cyanobacterium on agar. The phages from a single plaque were then suspended in CP buffer (Hu et al. 1981) and titered as described previously (Currier and Wolk 1979), on a variety of hosts.

Results

With the exception of ATCC strains 29413, 29106 and 29131, all of the strains listed in Table 1 contained isoschizomers of both endo R · *Ava*I (C↓PyCGPuG) (Hughes and Murray 1980) and endo R · *Ava*II (G↓G$\frac{A}{T}$CC) (Hughes and Murray 1980; Sutcliffe and Church 1978). Figure 1 illustrates this result. As noted above, Rosenvold and Szybalsky had observed that ATCC 29413 contains on isoschizomer, *Avr*I, of *Ava*I, and contains not *Ava*II but *Avr*II. We are unable to isolate *Avr*II.

Nostoc ATCC 29106 (PCC 7413) also contained two sequence-specific endonucleases, one of which was an isoschizomer of endo R · *Ava*II as we could show by comparing gel electrophoretic patterns of digests of the DNA of bacteriophage λ, SV40 and plasmid pAT153. We confirmed this conclusion by sequencing near the cleavage sites in SV40 at coordinates 557, 588 and 1018. We have named this *Ava*II isoschizomer *Nsp* (29106)II or *Nsp* (7413)II; in successive editions of Roberts' list (Roberts 1983) it will appear as *Nsp*HII. The second enzyme, *Nsp*HI was shown to be identical in its cleavage properties to an enzyme which we recently isolated from a different *Nostoc* strain *Nsp* (PCC 7524) or *Nsp*C and which specifically cleaves the nucleotide sequence PuCATG↓Py (Reaston et al. 1982). The identity of *Nsp* (7524)I and *Nsp* (7413)I is convincingly shown in Fig. 2. The specificity of *Nsp* (7413)I was also established directly by sequencing the oligonucleotides which border a cleavage site in bacteriophage M13 DNA. This is documented in Fig. 3.

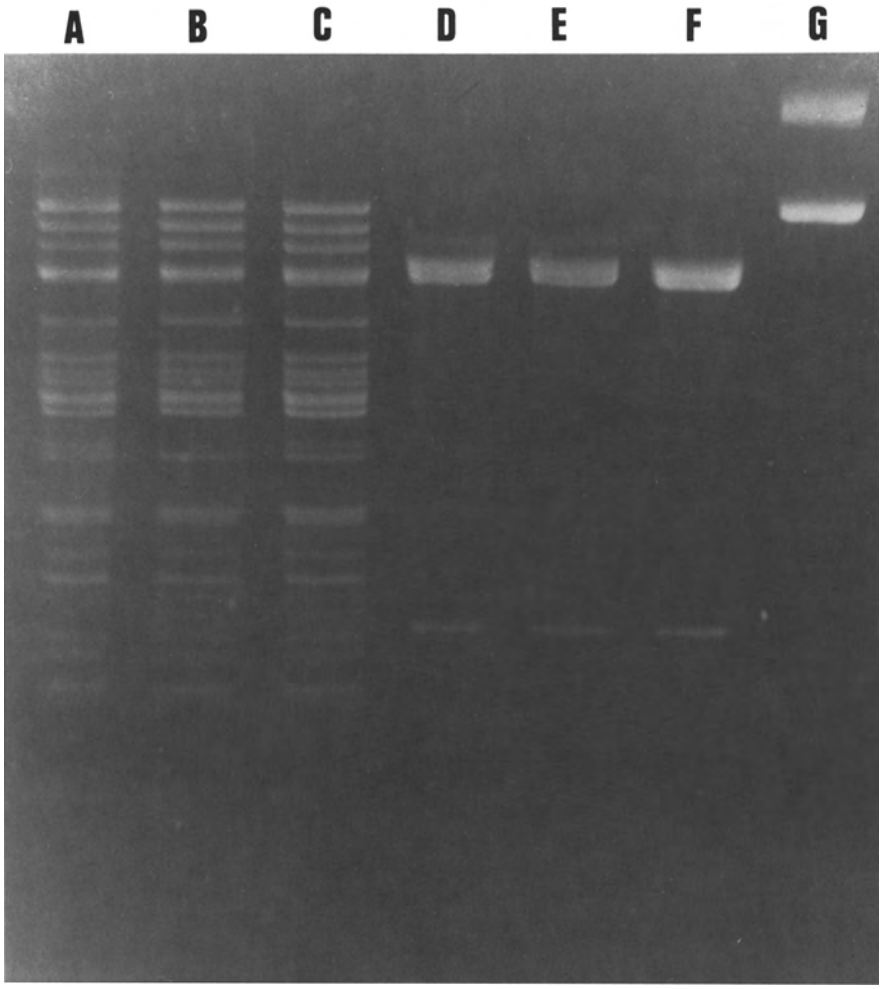

Fig. 2
Identity of cleavage patterns of bacteriophage λ DNA (A–C) and M13 DNA (D–G) by endo R · *Nsp* (7524)I and endo R · *Nsp* (7413)I (the sequence cleaved is PuCATG|Py). The former enzyme was present in lanes *A*, *C*, *D*, *F*, and the latter in lanes *B*, *C*, *E*, *F*. The enzyme from strain 7524 seems slightly contaminated [as is plausible from Reaston et al. (1982)]

Two restriction endonucleases were shown to be present in the last strain of the list of Table 1, *Nostoc* ATCC 29131 (PC 6705), but these deviated completely from all of the other strains. One was identified as an isochizomer of endo R · *Asu*II (de Waard and Duyvesteyn 1980) by a direct gel electrophoretic comparison of a digest of λ DNA. This enzyme was named *Nsp* (6705)I or *Nsp*BI.

An enzyme, *Nsp*BII, with cleavage properties hitherto not encountered was separated from *Nsp*BI on phosphocellulose. It was shown to be a single enzyme by chromatography on DEAE cellulose and by Fast Protein Liquid Chromatography on a Pharmacia apparatus in which proteins are eluted under high pressure as with HPLC. The enzyme *Nsp* (6705)II (= *Nsp*BII) was shown to cleave, specifically, the degenerate sequence $C\left(\frac{A}{C}\right)GC\left(\frac{T}{G}\right)G$. The experimental data to support this conclusion are as follows. SV40 DNA was cleaved with endo R · *Nsp* (6705)II; the resulting fragments were labeled in their 5′ termini with T4 polynucleotide kinase and subsequently cleaved with endo R · *Hae*III. Singly labeled fragments were separated on a polyacrylamide gel. Two of these were digested with pancreatic DNase; the resulting oligonucleotides were analyzed with the wandering spot method. Fig. 4A shows that one of these fragments terminates in 5′-C-T-G; it follows from the determined sequence (see legend) that the recognition site is CAG|CTG. A different fragment of which the sequence is documented in Fig. 4B also terminates in CTG. In this case the recognized nucleotide sequence is CCG|CTG. The complementary strand must have the structure CAG|CGG. These three hexanucleotides all fit the postulated recognition sequence given above.

A computer search of the sequences of ØX174 DNA and SV40 revealed that the postulated recognition sequence $C\left(\frac{A}{C}\right)CG\left(\frac{T}{G}\right)G$ was in complete accord with the observed cleavage pattern as shown by agarose gel electrophoresis in the presence of size markers.

In preliminary experiments, phage A-1(L), grown in *Anabaena* strain PCC 7120 and plated on various hosts or grown in those hosts and plated on them and on 7120; phage N-1, grown on *Anabaena* strains 7120, U. of Tokyo M-3 or U. Tokyo M-131 and plated on various hosts (but not *Anabaena* ATCC 29413); and phage AN-13 grown on *Anabaena* strains PCC 7120, Leningrad 458 (Myers), M-3, M-131 or ATCC 29151 and plated on various hosts gave no indication of differences in relative efficiency of plating which varied depending upon the host in which the phage had been grown. However, such differences were found for AN-22 (a phage which is not known to replicate in ATCC strains *Nostoc* sp. ATCC 29106, *Nostoc* sp. ATCC 29131 or *Anabaena variabilis* ATCC 29413): see Table 2.

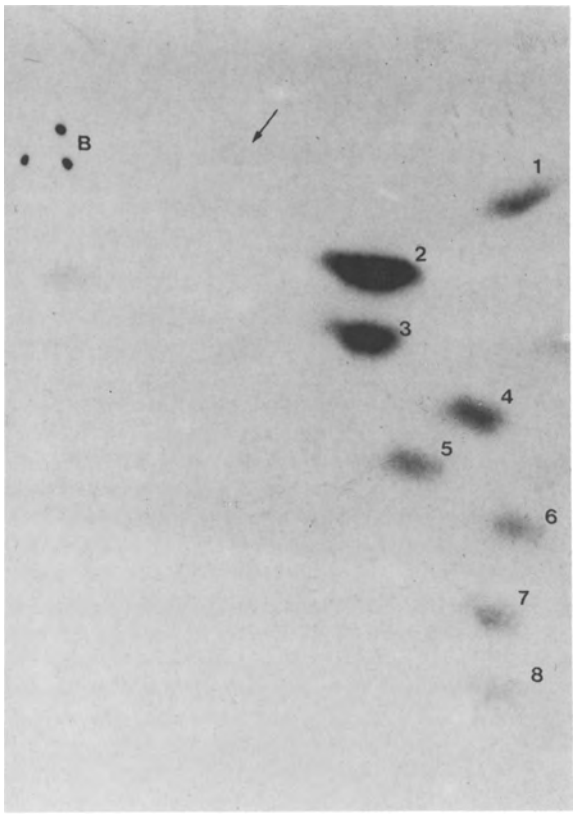

Fig. 3. Bacteriophage M13-oligonucleotides terminating in cleavage site (coordinates 3618–3623 (Van Wezenbeek et al. 1980) for endo R · *Nsp* (7413)I. The position of mononucleotide pT(3618) is indicated by an arrow. Labeled spots represent the sequence of the lower strand, coordinates 3618-3610. Spot *1* pTpT, spot *2* pTpTpC, spot *3* pTpTpCpA. Further additions in spots *4–8* are G, C, T, A, A. The blue marker is represented by *B*

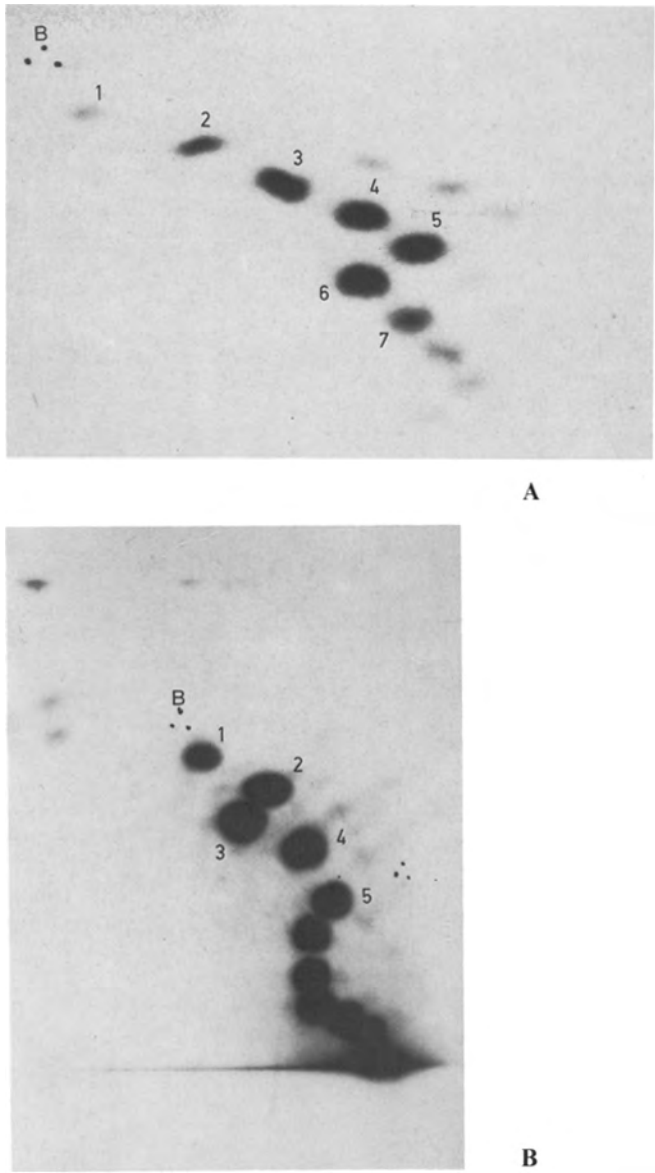

Fig. 4A, B. Proof that the sites of cleavage by endo R · *Nsp* (6705)II may be represented by $C\left(\frac{A}{C}\right)GlC\left(\frac{T}{G}\right)G$. **A** Wandering spot analysis of fragment of SV40 DNA terminating in the recognition site with coordinates 270–275. Mononucleotide pC (not visible) was identified by snake venom exonuclease digestion and paper electrophoresis. Spot *1* pCpT; spot *2* pCpTpG; spot *3* pCpTpGpG. Further additions to this sequence are T, T, C, T. **B** Wandering spot analysis of a fragment of SV40 DNA terminating in the recognition site with coordinates 662–667. Mononucleotide pC (not visible) was identified by snake venom exonuclease digestion and paper electrophoresis. Spot *1* pCpT; spot *2* pCpTpG; spot *3* pCpTpGpC. Further additions to this sequence are T, G, C, A, A, T, T

Discussion

Of the 64 cyanobacteria which were tested for lysis by 20 cyanophages of 7 groups (Hu et al. 1981), each of the hosts (Table 1), with three exceptions, was susceptible to all of the phages. Those three exceptions, ATCC strains 29106, 29131 and 29413 lysed by only 1, 1, and 3 of the phages, respectively, are the same ones which differ from the others in terms of the restriction endonuclease activities which we, and Rosenvold, could observe in vitro. It may therefore be useful to test whether genetic restriction is a significant aspect of the basis for these strains *not* being lysed by the other phages.

Phage AN-22, which lyses none of these strains, plated with widely varying relative efficiency on the other cyanobacteria tested, and the efficiency with which it plated was strongly dependent upon the host in which it had most recently been replicated. It is known that the Myers strain of *Anabaena variabilis* possesses restriction endonucleases *Ava*I, *Ava*II, and *Ava*III (Hughes and Murray 1980; Sutcliffe and Church 1978; Roizes et al. 1979), and we have shown directly that each of the hosts in Table 1 possesses enzymes which recognize the same nucleotide sequences as the first two of these nucleases. Strains 458, M-2 and M-3 are all thought to be derived from the Myers strain of *A. variabilits*, and indeed, all share with that strain the characteristics, unusual for an *Anabaena*, of forming short filaments and of being unable to form heterocysts.

We assume that, in general, in order to be attributable to restriction, a difference in relative efficiency of plating should be at least ten- to thirty-fold in magnitude (Arber and Linn 1969). With the exception that cyanophage AN-22 plates on M-2 with only about 5 % of the efficiency with which it plates

Table 2. Relative efficiency of plating of phage AN-22 on different hosts B after three cycles of growth on a host A

A	B:7120	458	M-2	M-3	M-131	*N. linckia*	27898	29105	29151
7120	1	0.015 (5)	0.014 (2)	0.037 (4)	0.79 (5)	0.64 (4)	0.0012 (4)	0.0054 (4)	0.0044 (3)
458	0.038 (4)	1	0.051 (3)		0.73 (3)			0.89 (3)	
M-2	I_1 (5)	1.21 (2)	1	0.70 (2)	0.67 (4)				1.12 (2)
M-3	0.29 (4)		1.91 (2)	1	0.68 (2)				
M-131	0.0090 (5)	0.0020 (3)	0.00085 (2)	0.00057 (2)	1	0.020 (2)	0.00079 (2)	0.0038 (2)	0.0016 (2)
N. linckia	I_2 (5)				1.94 (2)	1			
27898	1.37 (1)				1.46 (1)		1		
29105	I_3 (5)	1.40 (2)			3.38 (2)			1	
29131	0.037 (4)		0.24 (2)		1.44 (2)				1

The values given are the average of the results from a number of experiments shown in parentheses after each value. Inconsistent combinations I_1, I_2, and I_3 each gave values which were 0.024 or less and values in the range 0.48 – 0.90 (see Discussion)

on Leningrad strain 458 when grown in the latter strain, all of our data are consistent with the idea that strains 458, M-2, M-3, 29105, 29151, and perhaps 27898 share the same complement of restriction enzymes; it may be that, as suggested by the data for AN-22 grown in M-2, the phage absorbs less well to M-2 than to 458, rather than that AN-22 · 458 (i.e., AN-22 grown in 458) is restricted by M-2.

On the other hand, AN-22 · M-131 plates with much lower efficiency on any of the other strains than it does on M-131 whereas when grown in any of the other strains, AN-22 plates with comparable efficiency on M-131 and on the strain in which it was grown. The simplest interpretation of this result is that the *only* restriction endonucleases of M-131 are isoschizomers of *Ava*I and *Ava*II, whereas all of the other hosts have one or more additional restriction endonucleases. In particular, the fact that AN-22 · 7120 plates with much lower efficiency on 458, M-2, M-3, 29105 and 29151 than on 7120, whereas AN-22 458, AN-22 M-131, and AN-22 · 29151 plate with much lower efficiency on 7120 than on their hosts of origin is most simply explained by the assumption that 7120 has a restriction activity which differs from *Ava*I, II and III. That we failed to detect such an activity could be accounted for if it were present at too low a level to be detected with ca. 10 g fresh weight of cells; if it were unusually labile, if it were a Type I or Type III restriction endonuclease; or if differences in plating efficiency were controlled not by DNA-modification and restriction enzymes, but by a reversible alteration of the adsorption capacity of AN-22 on different hosts, as observed by Krüger et al. (1977, 1980) with coliphages T3 and T7.

The few data that we have for plating on *N. linckia* are generally consistent with the idea that the complement of restriction specificities in that strain is the same as the complement strain 7120. Unfortunately, AN-22 · *N. linckia* – as also AN-22 · M-2 and AN-22 · 29105 – plates with variable relative efficiency on 7120 and on the host of origin. We cannot with assurance identify the origin of such variability. One possible interpretation is that the physiological state of 7120, relative to infection by AN-22, is more sensitively affected by small changes in culture density than in the case with the other hosts with which we worked.

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