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A simple and rapid method for screening bacteria for type II restriction endonucleases: enzymes in *Aphanothece halophytica*

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Abstract. A method is described which allows a large number of bacterial strains to be rapidly and easily screened for the presence of site-specific endonucleases. The method involves selective permeabilization of the bacterial cell and analysis of the exuded material. Type II restriction endonucleases from cyanobacteria and Gram-negative eubacteria have been detected and new enzymes have been found. The method should be widely applicable and easy to modify for use in genera other than those tested. Three-site-specific endonuclease activities, detected by this method in *Aphanothece halophytica* PCC 7412, were purified and their recognition and cleavage specificities were determined *Aha*I and *Aha*II recognise and cleave the same DNA sequences as *Cau*II and *Acy*I respectively; the specificity of *Aha*III (TTT$^{\downarrow}$AAA) has been reported previously (Whitehead and Brown, 1982, FEBS Letters 143: 296–300).

Key words: Restriction-modification – Site-specific endonuclease – Cyanobacteria

Site-specific endonucleases are widely used in the analysis and restructuring of DNA molecules, and are used as model systems for the study of DNA-protein interactions. In spite of the fact that there are now nearly 500 such enzymes exhibiting about 100 different specifities (Roberts 1984) the isolation of novel restriction endonucleases is still required. These new enzymes provide useful new or alternative procedures for the molecular cloning of DNA and they generate new insights into the mechanisms whereby proteins interact with a specific sequence, or family of sequences, in DNA.

We have examined a number of bacterial strains for the presence of type II restriction endonucleases. There is no simple way to predict the bacterial strains in which these enzymes will be found. In vivo restriction has been observed in only a few species, and may be due to type I or type III enzymes (Arber 1979) as well as type II enzymes. A screening procedure must therefore use an in vitro method, the most obvious being the digestion of DNA with a crude endonuclease preparation and the analysis of the digestion products by gel electrophoresis. Discrete DNA fragment patterns indicate the presence of a site-specific endonuclease (Sharp et al. 1973). Previous methods of screening bacteria for type II restriction endonucleases have usually involved culture of bacteria on a moderate scale (> 100 ml), disruption of the harvested cells (e.g. by sonication, French press), and partial purification of the cell extract by column chromatography (Smith and Wilcox 1970; Green et al. 1978). Other methods which can be used with small-scale cultures of bacteria have been limited in the genera to which they are applicable (e.g. Mayer and Reichenbach 1978; Smith et al. 1976).

This paper describes a method of screening bacteria for site-specific endonucleases that is rapid; that requires only small quantities of cells; and that is applicable, with little or no modification, to a wide variety of bacterial genera. No method will be universally applicable and infinitely sensitive, but the method described here is based on the general properties of the bacterial envelope and will detect site-specific endonucleases that occur in reasonable yield in the presence of low or moderate amounts of non-specific nucleases and which are, therefore, good candidates for subsequent purification and characterization. We report here the purification and characterization. of three such endonucleases that were detected in *Aphanothece halophytica* PCC 7412.

Materials and methods

Bacteria and culture conditions

The bacterial strains used in this work are described in Table 1. *Anabaena* strains were grown in the liquid medium of Walsby and Brooker (1980) at 25° C under 1,500 lx illumination and were sparged with air. *Aphanothece halophytica* was grown in ASN-III medium of Rippka et al. (1979), modified to include 75 g NaCl/l, 6 g $MgCl_2 \cdot 6\,H_2O$/l 1.5 g KCl/l at 35° C under 500 lx illumination with occasional shaking. *Haemophilus* strains were grown in brain heart infusion supplemented with 10 mg haemin/l and 2 mg NAD^+/l, at 37° C with shaking. *Halobacterium salinarum* was grown in medium containing 250 g NaCl/l, 10 g $MgSO_4 \cdot 7\,H_2O$/l. 5 g KCl/l, 0.15 g $CaCl_2 \cdot H_2O$/l, 1 g glucose/l, 1 g casamino acids/l, at 35° C under 1,500 lx illumination with shaking. *Herpetosiphon giganteus* strains were grown in medium containing 3 g bactocasitone/l, 1 g yeast extract/l at 30° C with shaking. *Thermus aquaticus* was provided as frozen packed cells by Dr. A. Atkinson, CAMR, Porton Down, UK. All other strains were grown in nutrient broth (1 g/l) at 37° C with shaking.

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Abbreviations. Brij-58, 20 cetyl ether; Pu, purine nucleoside; Py, pyrimidine nucleoside

Table 1. Bacterial strains used in this work

Strain	Source/Reference
Agrobacterium tumefaciens C58	A. G. Hepburn
Alcaligenes sp PW101	This laboratory
Anabaena flos-aquae	CCAP 1403/13f
Anabaena subcylindrica	CCAP1403/46
Anabaena variabilis	ATCC 27892
Aphanothece halophytica	PCC 7412
Arthrobacter luteus	ATCC 21606
Bacillus globigii	Duncan et al. 1978
Enterobacter aerogenes PW201	K. Goverd
Enterobacter spp	K. Goverd
Haemophilus influenzae Rd exo$^-$	S. Goodgal
Haemophilus influenzae Rf	C. A. Hutchinson III
Halobacterium salinarum strain 5	H. Larsen
Herpetosiphon giganteus HFS101	H. Foster
Moraxella bovis	ATCC 10900
Nostoc muscarum	A. E. Walsby
Oscillatoria argardhii	A. E. Walsby
Proteus vulgaris	Bristol Public Health Lab.
Providencia stuartii 164	J. Davies
Pseudomonas facilis	M. van Montagu
Streptomyces albus G	J. M. Ghuysen
Streptomyces phaechromoges	F. Bolivar
Thermus aquaticus YT1	S. Sato

DNA and enzymes

These were prepared as described elsewhere (Brown et al. 1980) *Eco*RI was a kind gift of Dr. S. E. Halford. Bacteriophage λ DNA was digested with *Eco*RI and precipitated with ethanol prior to use in endonuclease assays; this made the detection of endonucleases with few target sites more simple. In common usage the term 'restriction endonuclease' is applied to site specific endodeoxyribonucleases even where there is no direct evidence for a restriction-modification system (Modrich and Roberts 1982).

Preparation of the cell-free extract

This method is derived from that of Godson and Sinsheimer (1967). A fresh bacterial pellet from 4 ml culture (obtained by multiple brief centrifugations in a 1.5-ml Eppendorf tube) was resuspended in 200 µl 25% (w/v) sucrose, 50 mM Tris-HCl, pH 7.5. Lysozyme (a fresh 1 mg/ml solution) and EDTA (500 mM, pH 8.0) were added to final concentrations of 0.1 mg/ml and 10 mM respectively. The mixture was incubated at 0° C for 2 min. $MgCl_2$ and Brij-58 (Sigma London Ltd.) were added in that order to the required final concentrations (50 mM and 0.5% respectively were optimal – see Results) and the sample was incubated at room temperature for 15 min. The sample was centrifuged at $10{,}000 \times g$ for 10 min at 40° C and the supernatant was assayed for endonuclease activity.

Endonuclease assay

Samples (1.5 µl) of the supernatant were incubated with *Eco*RI-cut bacteriophage λ DNA (0.25 µg) in 25 µl buffer R (50 mM NaCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.9) for 1–3 h at 37° C. Incubations with no added DNA ensured that any endogenous bacterial plasmids did not confuse interpretation of the results. Each reaction was stopped with 5 µl buffer S [15% (w/v) Ficoll, 50 mM EDTA, 0.03% (w/v) bromophenol blue] and were analysed by horizontal slab-gel electrophoresis in 1% (w/v) agarose in 90 mM Tris-borate, pH 8.3, 2.5 mM EDTA, 0.5 µg/ml ethidium bromide. Electrophoresis was normally at 10 V/cm for 2 h. The DNA was visualised on a long-wave UV transilluminator and the gels were photographed using Polaroid type 665 film, and a Schott-Jena KV470 filter.

Purification of restriction endonuclease activities from Aphanothece halophytica PCC 7418

Frozen packed cells of *Aphanothece halophytica* (stored at −70°C; 7 g) were resuspended in 10 ml 10 mM 2-mercaptoethanol, 10 mM Tris-Cl, pH 7.5 and sonicated (20 × 30 s; 60 W). After centrifugation at $100{,}000 \times g$ for 90 min the supernatant was made in 1 M in NaCl and applied to a column (50 × 2.0 cm) of Sepharose CL-6B. The column was eluted with 1 M NaCl, 10 mM 2-mercaptoethanol, 10 mM Tris-Cl, pH 7.5 and fractions (5 ml) were assayed for specific endonuclease activity as described elsewhere (Brown et al. 1980). Fractions containing endonuclease activity were pooled and dialysed against PC buffer [10 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% (w/v) glycerol, 10 mM potassium phosphate, pH 7.0]. The endonuclease activities were subsequently fractionated by column chromatography on phosphocellulose P11 (20 cm × 2 cm), DEAE-cellulose (10 cm × 2 cm) and heparin-sepharose (3 cm × 1.5 cm) using gradients (200 ml) of 0–1.0 M KCl in PC buffer. Active fractions which apparently contained a single endonuclease activity were dialysed against PC buffer containing 50% glycerol and stored at −20°C. The final yields of endonucleases were variable between preparations, but were typically, per g packed cells: 150 units *Aha*I, 100 units *Aha*II, 150 units *Aha*III.

Characterization of the cleavage of AhaI and AhaII

The recognition and cleavage specificities were determined by the method of Brown and Smith (1980). The numbers of cleavage sites for each enzyme on a number of fully-sequenced DNA molecules were determined both in single digests and in digests with a second enzyme. These data were then compared with the table of Fuchs et al. (1980) in order to indicate possible recognition sequences. Single-stranded templates of bacteriophage M13mp7 DNA, or a recominant M13mp7 DNA were constructed by standard methods (Sanger et al. 1980). The phosphodiester bonds cleaved by the restriction endonuclease were determined by the method described elsewhere (Brown and Smith 1980; Whitehead and Brown 1982).

Results

Detection of known restriction endonuclease activities

Cell-free extracts were prepared from three bacterial strains known to contain restriction endonuclease activities using a variety of Brij-58 and $MgCl_2$ concentrations. These strains were *Alcaligenes* sp. PW101 (*Asp*AI; Whitehead and Brown, unpublished data), *Providencia stuartii* 164 (*Pst*I, Smith et al. 1976) and *Bacillus globigii* (*Bgl*I and *Bgl*II, Duncan et

Table 2. Screening of strains reported to contain type II restriction endonucleases by the Brij/$MgCl_2$ permeabilization procedure

Strain	Restriction endonucleases	Result[a]
Agrobacterium tumefaciens C58	*Atu*CI	(−)
Alcaligenes sp	*Asp*AI	+
Anabaena subcylindrica	*Asu*I, *Asu*II, *Asu*III	+
Anabaena variabilis	*Ava*I, *Ava*II, *Ava*III	+
Arthrobacter luteus	*Alu*I	+
Bacillus globigii	*Bgl*I, *Bgl*II	+[b]
Haemophilus influenzae Rd	*Hind*II, *Hind*III	(+)
Haemophilus influenzae Rf	*Hinf*I	(+)
Herpetosiphon giganteus HP1023	*Hgi*AI	−
Herpetosiphon giganteus HP1049	*Hgi*HI, *Hgi*HII, *Hgi*III	−
Herpetosiphon giganteus HFS 101	*Hgi*JI, *Hgi*JII	−
Moraxella bovis	*Mbo*I, *Mbo*II	(+)
Proteus vulgaris	*Pvu*II	(−)
Pseudomonas facilis	*Pfa*I	(−)
Streptomyces albus G	*Sal*GI	−
Streptomyces phaechromogenes	*Sph*I	−
Thermus aquaticus YT1	*Taq*I	(+)

[a] + indicates the detection of specific bands following agarose gel electrophoresis of the samples. ( ) indicates the presence of non-specific nuclease which makes the detection of specific bonds difficult after longer incubation times

[b] Only one cleavage site was detected in the *Eco*RI-cut λ DNA substrate

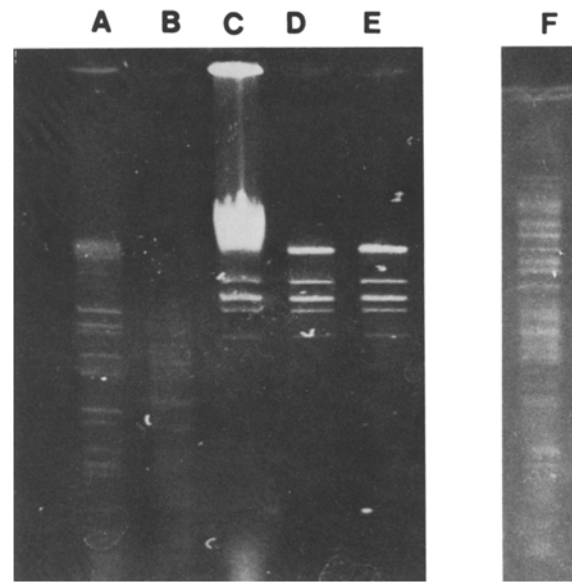


Fig. 1. Screening for site specific endonuclease activity in Brij/$MgCl_2$ supernatants of several bacterial strains. Electrophoresis of samples *A* to *E* was longer than that of sample *F*. *A Anabaena variabilis* (positive control). *B Anabaena flos-aquae*. *C Halobacterium salinarum* strain 5. *D* and *E Enterobacter* spp. *F Aphanothece halophytica*

al. 1978). In all three cases optimum release of site-specific endonuclease activity was at 0.5% (w/v) Brij-58 and 50–100 mM $MgCl_2$. Higher Brij-58 concentrations or low (< 10 mM) $MgCl_2$ concentrations cause release of other macromolecules from the cell (e.g. chromosomal DNA) which interfere with the assay. Low Brij-58 concentrations or higher $MgCl_2$ concentrations (> 100 mM) cause inefficient release of endonuclease activity.

Other restriction endonuclease-producing strains were treated with 0.5% Brij-58, 50 mM $MgCl_2$ as described in Materials and methods. A list of strains tested and the result of the assays is given in Table 2.

Assays for new restriction endonuclease activities

Several strains of cyanobacteria and members of the family *Enterobacteriaceae* have been surveyed by this method, as have other individual species. Figure 1 shows the result of such an assay. Site-specific endonuclease activities were detected in the cyanobacteria *Anabaena flos-aquae* (channel B) and *Aphanothece halophytica* (channel F). A restriction endonuclease-producing strain of *Anabaena variabilis* (channel A) was used as a positive control.

The halophilic archaebacterium *Halobacterium salinarum* strain 5 is osmotically sensitive and was completely lysed in the low-salt buffers used in this experiment. DNA and RNA were released, but no specific endonucleases were detected (Fig. 1, channel C). The *Enterobacter* strains tested showed no signs of site-specific endonuclease activity. Long incubations of these preparations with DNA showed that non-specific nucleases had been released by Brij-$MgCl_2$ treatment, indicating that the cells had been permeabilized.

Table 3. Minimum number of cleavage sites for *Aha*I, *Aha*II and *Aha*III on several DNAs of known sequence[a]

DNA	Enzyme		
	*Aha*I	*Aha*II	*Aha*III
pBR322	9 (10)	5 (6)	2 (3)
φX 174 RF	1 (1)	7 (7)	2 (2)
M13mp9 RF	3 (3)	1 (1)	5 (5)
SV40	0 (0)	0 (0)	12 (12)
lambda	> 50 (111)	> 35 (49)	13 (13)

[a] These are minimum values because on electrophoretic analysis two or more closely spaced sites may appear as a single site and in complex digests the exact number of DNA fragments produced cannot be counted. The numbers of cleavage sites for each enzyme predicted by computer analysis (Staden 1977) are given in parentheses

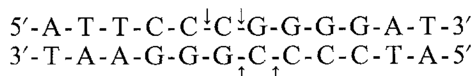
Purification of restriction endonucleases from Aphanothece halophytica PCC 7412

Three separate endonuclease activities were isolated from *Aphanothece halophytica*. *Aha*I eluted from phosphocellulose P11 at 0.5–0.7 M KCl, and from DEAE-cellulose at 0.1–0.25 M KCl: *Aha*II eluted from phosphocellulose P11 at 0.3–0.5 M KCl and from DEAE-cellulose at 0.2–0.4 M KCl; *Aha*III eluted from phosphocellulose P11 at 0.3–0.4 M KCl (with *Aha*II), and from DEAE-cellulose at 0.35–0.6 M KCl. *Aha*III was further purified on heparin-sepharose and eluted at 0.1–0.45 M KCl. Two-column purification was sufficient for identification of the cleavage specificity of the enzymes. The novelty of the *Aha*III site (Whitehead and Brown 1982) provided the incentive to obtain the more highly-purified preparation of this enzyme.

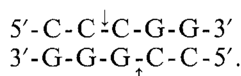
Characterization of the cleavage specifities of AhaI, AhaII and AhaIII

The minimum numbers of cleavage sites for *Aha*I, *Aha*II and *Aha*III on a variety of DNAs are shown in Table 3. Examination of computer-generated tables (Fuchs et al. 1980) and approximate mapping of the sites of cleavage of the enzymes in double-digests indicated that *Aha*I recognised the sequence 5′-CC$^{C}_{G}$GG-3′; that *Aha*III recognised the family of sequences 5′-GPuCGPyC-3′; and that *Aha*III recognised the sequence 5′-TTTAAA-3′. Details of the determination of the *Aha*III site have been published (Whitehead and Brown 1982).

The cleavage site of *Aha*I within the sequence 5′-CC$^{C}_{G}$GG-3′ was determined using the sequence in the multiple cloning site of M13mp9. There are two overlapping recognition sites in the sequence:

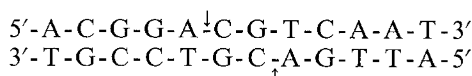


and cleavage is as indicated by the arrows. Therefore, *Aha*I cleaves within its recognition sequence:

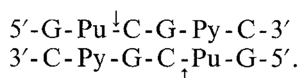


In some preparations of *Aha*I another site-specific endonuclease activity could be detected due to the great sensitivity of the site-location method. This activity was unstable and could not be fully characterised, but it is not *Aha*II or *Aha*III, and probably represents a fourth type II restriction endonuclease in *Aphanothece halophytica* PCC 7412.

The cleavage site of *Aha*II within the sequence 5′-GPuCGPyC-3′ was determined using a recombinant M13mp7 DNA containing the *Aha*II site at position 1288 of transposon Tn501 DNA (Misra et al. 1984). *Aha*II cleaved at the sequence:



indicating, in conjunction with the mapping data, that the recognition and cleavage site of *Aha*II is:



Discussion

The method described here has been shown to work with a number of different genera of cyanobacteria and Gram-negative eubacteria (Table 2), and the method may work well in other genera which have not been tested. The limits of the method are best ascertained from those strains which are not susceptible. The enzymes *Pvu*II, *Atu*CI and *Pfa*I could not be detected after treatment of their host strains, even though non-specific nuclease activity could be detected in all three cases (Table 2). We suggest that this is because of the low yield of these enzymes and the relatively high amounts of non-specific endonuclease in these strains. Using sonication methods on gram quantities of cell paste we have been unable to detect *Atu*CI or *Pfa*I activity even after purification of the sonicates, suggesting that these enzymes may be unstable.

No evidence of cell permeabilization could be seen with two strains of *Streptomyces* nor with five strains of *Herpetosiphon giganteus* (Table 2). Microscopic examination indicated that the lysozyme treatments has not caused spheroplast formation in these cases. The tendency of *Streptomyces* and *Herpetosiphon* cells to grow in a non-dispersed fashion generates a culture in which the cells are in a variety of stages of growth. This has been proposed to be the main obstacle to obtaining a uniform treatment for the formation of spheroplasts in *Streptomyces* (Hopwood 1981). *Herpetosiphon* cells are surrounded by a sheath (Holt and Lewin 1968), which may make them resistant to lysozyme etc. At the other extreme, *Halobacterium salinarum* strain 5 was osmotically ruptured by the protocol used here, and the macromolecular contents of the cells were released.

The limitations of the method are therefore in the susceptibility of the cells to spheroplast formation and in the presence of a high ratio of site-specific endonuclease to nonspecific nuclease activities. The latter limitation need not detract from the usefulness of the method, as the method is designed merely to search for novel type II restriction endonucleases which are suitable for purification and subsequent use in the restructuring and analysis of DNA. It is not designed to provide an exhaustive catalogue of type II restriction endonucleases in a given genus. The method has the advantage that it is applicable to a variety of genera, that it is rapid and that it requires only small-scale cultures.

Among the species that were tested for the first time no site specific endonuclease activities were detected under these conditions in five separate isolates of *Enterobacter,* two strains of *Nostoc muscarum,* two strains of *Oscillatoria argardhii,* and one strain of *Halobacterium salinarum.* This does not preclude the presence of very low levels of such activities in these strains, nor the presence of unstable activities.

The Brij-lysis method and the modified site-location method (Brown and Smith 1980; Whitehead and Brown 1982) were used to characterise three restriction endonucleases from *Aphanothece halophytica* PCC 7412. Evidence was obtained for a further activity which has not been characterised. *Aha*III was the first enzyme discovered which has an all A-T base pair recognition sequence, and the only other such enzyme (*Dra*I; Purvis and Moseley 1983) recognises and cleaves the same DNA sequence. The two enzymes *Aha*I and *Aha*II have specificities identical with other enzymes from photosynthetic bacteria, respectively *Cau*II (from *Chloroflexus auranticus*) and *Acy*I (from *Anabaena cylindrica*). It is not unusual for restriction endonucleases of identical specificity to be found in a number of different bacterial genera and in a number of species without a genus. Strains containing a number of different endonucleases are common in the cyanobacteria, and some specificities (e.g. those recognising the same sites as *Acy*I, *Ava*II, *Asu*I, *Asu*II, *Cau*II) are found in a variety of permutations within the cyanobacteria. This may indicate that some of the activities are plasmid borne; we have no good evidence for the presence or absence of plasmids in *Aphanothece halophytica* PCC 7412.

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