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Characterization of Additional Host Restriction-Modification Systems in the Unicellular Cyanobacterium *Cyanothece* sp.

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In order to develop a gene transfer system for the unicellular diazotrophic cyanobacterium *Cyanothece* sp. strain BH68K, this organism has been further investigated for the presence of additional host restriction-modification enzymes other than *Csp*68KI, previously reported for *Cyanothece* sp. Analysis of cell extracts by phosphocellulose and Mono Q fast protein liquid chromatography (FPLC) has led to the identification of three new restriction endonucleases. These enzymes have been designated *Csp*68KII, *Csp*68KIII, and *Csp*68KVI. *Csp*68KII is an isoschizomer of *Asu*II and restricts DNA at the recognition sequence 5′-TT/CGAA-3′. Cleavage occurred between thymine and cytosine producing 2 bp 5′ overhang ends. The third restriction endonuclease, *Csp*68KIII, is an isoschizomer of *Ava*III and restricts DNA at the recognition sequence 5′-ATGCA/T-3′. Cleavage occurred between the 3′ adenosine and thymine nucleotides producing 4 bp 3′ overhang ends. The fourth enzyme identified, *Csp*68KVI, recognizes CGCG and cleaves this sequence between the internal guanine and cytosine nucleotides producing blunt ends. © 1996 Academic Press, Inc.

Cyanothece sp. is a marine unicellular cyanobacterium which is able to perform oxygenic photosynthesis and reduction of dinitrogen to ammonia in a single cell without any apparent morphological differentiation [1]. The enzyme nitrogenase which catalyzes nitrogen fixation is rapidly inactivated when exposed to oxygen [2,3]. Since this organism is capable of carrying out photosynthesis and nitrogen fixation simultaneously, *Cyanothece* sp. must have developed some method(s) of protecting nitrogenase from oxygen inactivation [1,4]. Under N_2-fixing conditions, *Cyanothece* sp. has a growth rate which is significantly faster than many other N_2-fixing, unicellular cyanobacteria (i.e. doubling times of 16–20 h vs. 20–100 h), and can form colonies on solid agar medium, which makes this organism amenable to standard molecular genetic procedures [1,5]. However, in order to study the molecular biology of photosynthesis and nitrogen fixation in *Cyanothece* sp., a system for efficient gene transfer is essential.

In the course of developing a gene transfer system for *Cyanothece* sp., a small 4.8 kb plasmid, pSE480, was isolated from the clonal isolate BH68F [6]. This plasmid has been used to construct several shuttle vectors to be used in the transformation of *Cyanothece* sp. strain BH68K, which lacks pSE480 [6,7]. Initial transformation experiments using natural competency and electroporation proved unsuccessful. A large number of both filamentous and unicellular species have been shown to contain one or more type II sequence-specific restriction endonucleases (REases) [8]. Each type II REase is found in close association with a modification enzyme counterpart which recognizes and methylates the same base sequence at either adenosine or cytosine nucleotides [9]. The modification enzyme methylates the host genome in order to protect it from its concomitant restriction enzyme. The purpose of such an elaborate system in these organisms is believed to be to provide protection from exogenous DNA which may be deleterious to them. Therefore, *Cyanothece* sp. chromosomal DNA was examined for evidence of methylated nucleotides, and cell extracts were assayed for REase activity. Evidence for both was found, indicating that host restriction-modification (HRM) is impeding gene transfer in *Cyanothece* sp. [5].

The first HRM system characterized in *Cyanothece* sp. was *Csp*68K1, which is an isoschizomer

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of *Ava*II [5]. The present paper describes the identification of three additional HRM systems using chromatographic techniques. Two six-base-pair, and one four-base-pair recognition enzymes have subsequently been purified, and their recognition and cleavage sites identified.

MATERIALS AND METHODS

Culture growth and cell extract preparation. Cyanothece sp. BH68K was grown in nitrate-containing medium AN^+ as described previously [5]. The cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml of medium AN^+, while being shaken at 100 rpm under a light intensity of 30–40 microeinsteins/m^2/s. Crude extract for restriction enzyme activity was prepared as described previously [5]. For large-scale chromatographic purification, cultures of *Cyanothece* sp. BH68K were grown in 4-liter aspirator bottles containing 3 liters of medium AN^+. These cultures were maintained at 30°C under a light intensity of 90 microeinsteins/m^2/s and were continuously stirred. Cells were harvested from 1500 ml of culture having an optical density (OD_{750} of 1.0 and pelleted at 6000×g for 10 min. Pellets were divided into aliquots of 1 g wet weight and were stored at −70°C for later use.

Frozen cell pellets (1 g, wet weight) were resuspended in 3 ml of potassium phosphate buffer [20 mM potassium phosphate, 7 mM β-mercaptoethanol, 0.144 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylene diamine tetraacetic acid (EDTA), pH 7.0], and lysozyme was added at a final concentration of 3 mg/ml (w/v). The resulting suspension was incubated in a 15 ml centrifuge tube for 5 hours at 37°C with gentle mixing on a nutator. Cells were broken by vortexing 0.75-ml aliquots of the mixture with 0.45 g of glass beads (diameter: 50–100 μm) in four 1.6 ml microcentrifuge tubes for 1 hour. This was followed by centrifugation (14,000Xg) for 10 minutes at 4°C. The resulting supernatants were again vortexed with 0.25 g of glass beads for an additional 10 minutes and centrifuged for the same duration to remove nucleic acids. Supernatants were pooled and subjected to ultracentrifugation at 104,000×g for 30 minutes at 4°C to remove any remaining particulate matter. A 2 ml crude cell extract was subsequently recovered from this procedure.

Chromatographic techniques. The ion exchange chromatographic techniques were similar to those described previously [10,11] with the following modifications. The crude cell extract was loaded onto a phosphocellulose (Whatman) cation-exchange column (1 × 17.5 cm) which had been previously equilibrated with potassium phosphate buffer. The column was developed with a linear gradient of NaCl (0–1 M, 140 ml) for 140 minutes at a rate of 1 ml/minute. One ml fractions were collected and tested for restriction enzyme activity by digesting 500 ng lambda DNA with 2 μl of fraction eluant in a 20 μl reaction volume. Reactions were supplemented with REact Buffer 1 (GibcoBRL) and incubated for 2 hours at 50°C. Fragments were then separated by agarose gel electrophoresis on 1% (w/v) agarose gels at 100V. Groups of fractions exhibiting restriction activity were pooled and concentrated using Amicon Centriprep-10 concentrators (10,000 MW cutoff). Each pool was dialyzed against a Tris-glycerol buffer [20 mM Tris-HCl, pH 7.4, containing 5% (v/v) glycerol] to remove salts. One pool, designated pool 1 exhibited more than one restriction enzyme activity, and was further subjected to FPLC. After concentration and dialysis pool 1 was loaded onto a Pharmacia MonoQ anion-exchange column (5 × 50 mm; 1 ml) which had been equilibrated with Tris-glycerol buffer [20 mM Tris-HCl, pH 7.4, containing 5% (v/v) glycerol]. The column was developed with a linear gradient of NaCl (0–0.9 M) for 25 minutes at a flow rate of 1 ml/min and 0.5 ml fraction were collected. Fractions were tested for restriction activity as previously described.

Restriction endonuclease activity. E. coli plasmids used in this study were isolated from *E. coli* XL1-Blue MRF cells (Stratagene). Restriction enzymes and modification enzymes were obtained from Boehringer Mannheim, GibcoBRL, Promega, New England Biolabs., Inc. (NEB) and United States Biochemical, Inc. (USB).

Enzyme activity was assayed by incubating 10 μl of column purified or crude cell extract with 500 ng plasmid or lambda DNA (Boehringer Mannheim) in a 20 μl reaction volume at either 37°C or 50°C for 2 hours. Reactions were supplemented with standard commercial restriction buffers (GibcoBRL) and reactions were performed using a range of different salt concentrations. Plasmids used in this analysis were pBC(SK^-) (Stratagene), pACYC184 [12], pJCF22 [13] and pKMS3. The latter molecule, pKMS3, is a pUC19 [14] derivative containing the methylase gene, M. *Sin*I, from pΔHH2 [15,16], which methylates *Ava*II sites. The plasmid pKMS3 also contains *psb*A::Tn5 (Km^r/Nm^r) from pRL448 (17). This plasmid, pKMS3, was methylated with M. *Cvi*BIII ($TCG^{m6}A$) (Boehringer Mannheim) by incubating it for 2 hours at 37°C in the presence of 160 μM S-adenosylmethionine (SAM). The methylated plasmid was then divided into three aliquots, and each aliquot was restricted with either *Xba*I, *Eco*RV or *Nde*I. The restricted DNA from each of these samples was subsequently divided into two equal aliquots and treated with either *Nsi*I (an isoschizomer of *Ava*III) or *Cyanothece* cell extract. Fragments were separated by agarose gel electrophoresis to map restriction sites and to compare *Cyanothece* restriction activity with *Nsi*I. Agarose gel electrophoresis was carried out on 0.7% agarose gels using Tris-acetate running buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).

Sequence specificity analysis. Analysis of the cleavage specificity of REases Csp68KII (*Asu*II) and *Csp*68KIII (*Ava*III) was based on the strategy of Brown and Smith [18]. This procedure has been adapted for the analysis of REases in *Cyanothece* sp. and has been described elsewhere [5]. For analysis of *Csp*68KII, the primer KMNM5 (5′-ATAGAAGGCGGCGGTGGAA-3′) was designed. This primer hybridizes downstream to the Tn5 (Km^r/Nm^r) gene of pKMS3 44 bp from an *Asu*II (TTCGAA) site. Similarly, the primer MSINI2 (5′-CTCGCTAGTAGGGTTT-3′) was constructed for the analysis of *Csp*68KIII, and this primer hybridizes to the M. *Sin*I gene of pKMS3 186 bp away from an

*Ava*III (ATGCA) site [12]. Extension reactions were performed as described previously in the presence of [α^{35}S]dATP [5]. The labeled samples were extended from the KMNM5 primer in the presence of 80 μM each of dATP, dCTP, dGTP and dTTP and were subsequently treated with either *Nsp*V (an isoschizomer of *Asu*II), or the partially purified *Csp*68KII for 1.5 hours at 50°C. The labeled samples extended from the MSINI2 primer were treated with either *Nsi*I or cell extract containing *Csp*KIII for 3 hours at 37°C. The digested samples from the above four reactions were then divided into two equal aliquots. One aliquot from each of the *Nsp*V and *Csp*68KII restricted samples was treated with Klenow fragment (GibcoBRL) for 10 minutes at 37°C to fill in staggered ends. One aliquot from each of the *Nsi*I and *Csp*68KIII restricted samples was treated with T4 DNA Polymerase (USB) for 10 minutes at 37°C to remove 3′ over-hang ends. The samples extended from the KMNM5 primer were denatured and run on an 8% polyacrylamide gel in parallel to dideoxy chain termination sequencing reactions extended from the same primer. The samples extended from the MSINI2 primer were denatured and run on a 6% polyacrylamide gel in parallel to dideoxy chain termination sequencing reactions extended from the MSINI2 primer. For the analysis of *Csp*68KVI, a universal primer (USB) was hybridized to pUC19 293-bp downstream from an *Fnu*DII (CGCG) site. Primer extensions using Sequenase (USB) were performed in the presence of [α^{35}S] dATP and 80 mM each of dCTP, dGTP, and dTTP. Samples were divided into two aliquots which were then treated with either *Bst*UI (an isoschizomer of *Fnu*DII) or *Csp*68KVI for 1.5 hours at 37°C. These samples were then denatured and run on a 6% polyacrylamide gel in parallel to dideoxy chain termination sequencing reactions extended from the same primer.

RESULTS

Identification of Csp68KII, Csp68KII, and Csp68KVI Activity

Fractions eluted from the phosphocellulose column showed restriction activity in two pools when lambda DNA was used as a substrate. These were designated as pools 1 (fractions 60 to 80) and 2 (fractions 105 to 120). Pool 1 activity was eluted from 0.27 to 0.43 M NaCl, whereas pool 2 activity was seen from 0.63 to 0.74 M NaCl. Pool 1 fractions were further characterized by subjecting them to FPLC Mono Q anion-exchange chromatography. *Csp*68KI eluted between 0.20 and 0.26 M NaCl and *Csp*68KII eluted from 0.34 to 0.41 M NaCl (data not shown). The *Csp*68KI activity in FPLC fractions was confirmed based on the cleavage pattern obtained with pUC19 and pBR322 DNA [5].

A particular restriction pattern was detected when purified *Csp*68KII was incubated at 50°C using 50mM Tris HCl (pH 8.0), 10 mM $MgCl_2$. Physical mapping of restriction fragments released from DNA substrates indicated that this restriction activity corresponded to *Asu*II (TTCGAA). Comparison of DNA restricted with *Nsp*V (*Asu*II) or purified *Csp*68KII revealed an identical restriction pattern (Fig. 1). The enzyme responsible for this activity from *Cyanothece* sp. strain BH68K has been designated *Csp*68KII.

When DNA was incubated with *Cyanothece* sp. strain BH68K crude cell extracts in the presence

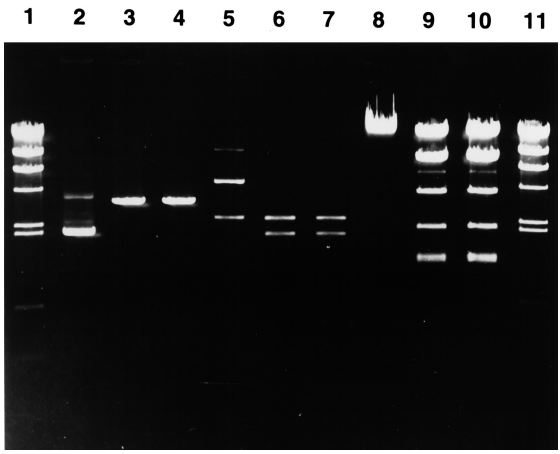

FIG. 1. Agarose gel electrophoresis of DNA digested with either *Nsp*V or *Csp*68KII, Lanes 1 and 11: λ-*Hind*III digests as markers, lanes 2–4: pBC(SK-) untreated, *Nsp*V, and *Csp*68KII treated, lanes 5–7: pACYC184 untreated, *Nsp*V, and *Csp*68KII treated, lanes 8–10: λ DNA untreated, *Nsp*V, and *Csp*68KII treated.

of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl at 37°C, a third enzymatic activity was observed, which coincided with that of *Ava*III. In order to clearly map the restriction sites at which this enzyme restricts DNA, pKMS3 (Fig. 2A) was first methylated with M. *Cvi*BIII which methylates TCG^{m6}A recognition sites. This enzyme will methylate the adenosine in the *Asu*II recognition site (TTCG^{m6}AA) and potentially provide protection from restriction, although the methylation specificity of M. *Csp*68KIII was previously unknown. The methylated plasmid was then divided into three aliquots and digested with *Xba*I, *Eco*RV, and *Nde*I. These samples were subsequently further subdivided and digested with either *Nsi*I or strain BH68K cell extract. Comparison of DNA fragments generated by the *Nsi*I (*Ava*III) and cell extract-treated samples showed an identical restriction pattern (Fig. 2B), and the fragment sizes correctly matched the restriction map of pKMS3. The background smear found in lanes digested with cell extract is believed to be due to sheared chromosomal DNA not completely removed during preparation of the crude extract, or

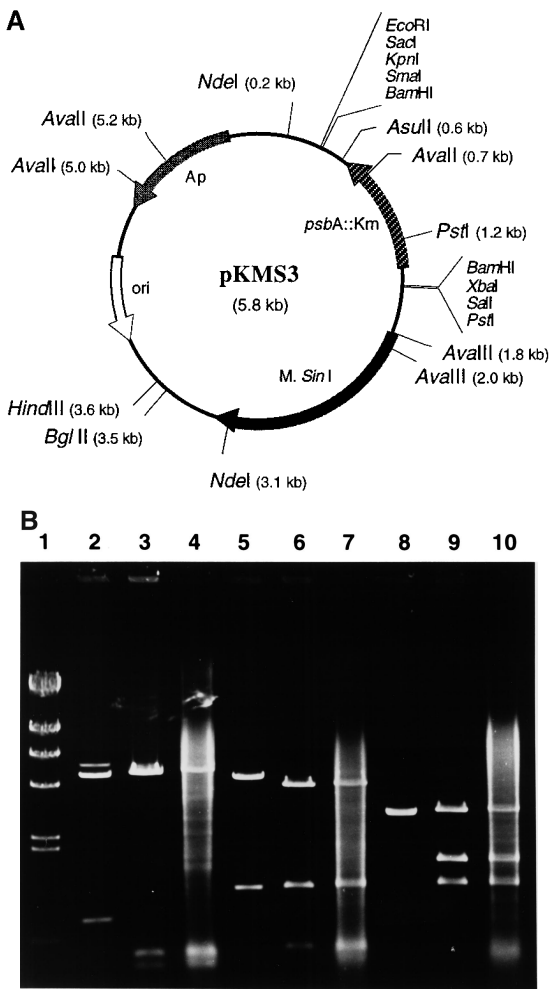

FIG. 2. (A) Restriction map of pKMS3. This plasmid was constructed from pUC19 by the insertion of M. *Sin*I and *psb*A::KmNm (npt) into the multiple cloning site. The M. *Sin*I gene methylates all *Ava*II sites and therefore also inhibits restriction by *Csp*68KI. (B) Agarose gel electrophoresis of pKMS3 digested with *Xba*I, *Eco*RV, or *Nde*I followed by digestion with either *Ava*III or *Csp*68KIII. Lane 1: λ-*Hind*III digest as marker, lanes 2–4: pKMS3 digested with *Xba*I, *Xba*I/*Ava*III, and *Xba*I/*Csp*68KIII, lanes 5–7: pKMS3 digested with *Eco*RV, *Eco*RV/*Ava*III, and *Eco*RV/*Csp*68KIII, lanes 8–10: pKMS3 digested with *Nde*I, *Nde*I/*Ava*III, *Nde*I/*Csp*68KIII.

it may be due to low level activity of a four-base-recognizing restriction enzyme. This enzyme activity from *Cyanothece* sp. strain BH68K has subsequently been designated *Csp*68KIII.

After concentration and dialysis in Tris-glycerol buffer, the restriction enzyme in pool 2 at medium salt conditions [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$] and at 37°C generated distinct bands. The pool 2 restriction pattern on various DNA substrates matched with patterns generated by *Bst*UI (*Fnu*DII isoschizomer) (Fig. 3). This activity in pool 2 has been designated *Csp*68KVI.

Cleavage Specificity of Csp68KII, Csp68KIII, and Csp68KVI

The cleavage specificity of *Cyanothece* sp. strain BH68K REases *Csp*68KII and *Csp*68KIII are shown in Figure 4. In the case of *Csp*68KII, panel A shows the control samples treated with *Nsp*V and *Csp*68KII. Comparison of the bands obtained show that these restriction endonucleases recognize the same DNA sequence and cleave the DNA at exactly the same position. The labeled bands generated from restriction activity alone co-migrate with the 3′-T of the recognition sequence 5′-TTCGAA-3′ (Fig. 4A). Treatment of the restricted DNA with Klenow fills in the two-nucleotide recessed ends generating bands two nucleotides greater in length (Fig. 4A).

*Csp*68KIII cleavage specificity is shown in Figure 4B which shows control samples treated with *Nsi*I followed by T4 DNA Polymerase and *Nsi*I alone. Figure 4B also shows the *Csp*68KIII followed by T4 DNA Polymerase and *Csp*68KIII. Restriction alone generated a labeled band that co-migrates with the 3′-A of the labeled strand complementary to the sequence 5′-ATGCAT-3′. Treatment with T4 DNA Polymerase removes four nucleotides and labeled bands were observed co-migrating with the 5′-A of the labeled strand complementary to the sequence 5′-ATGCAT-3′ (Fig. 4B).

The cleavage specificity of *Csp*68KVI was examined using primer extension analysis as described above. A universal primer (USB) was annealed to pUC19 and extended toward an *Fnu*DII site in the presence of [α^{35}S]dATP yielding labeled double-stranded DNA. Upon the restriction of the labeled DNA, both *Bst*UI (an *Fnu*DII isoschizomer) and *Csp*68KVI generated 293-bp fragments (results not shown) resulting from blunt end cleavage of the cite CGCG between the internal

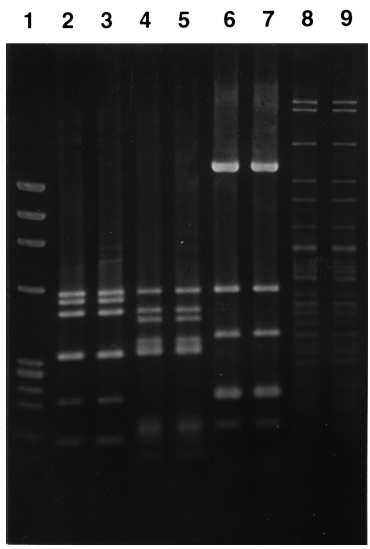

FIG. 3. Agarose gel electrophoresis of plasmid and lambda DNA treated with either *Bst*UI or *Csp*68KVI. Lane 1: ØX174 RF DNA digested with *Hae*III, lanes 2,4,6,8: pUC19, pBR322, pJCF22, or lambda DNA treated with *Bst*UI, lanes 3,5,7,9: pUC19, pBR322, pJCF22, or lambda DNA treated with *Csp*68KVI.

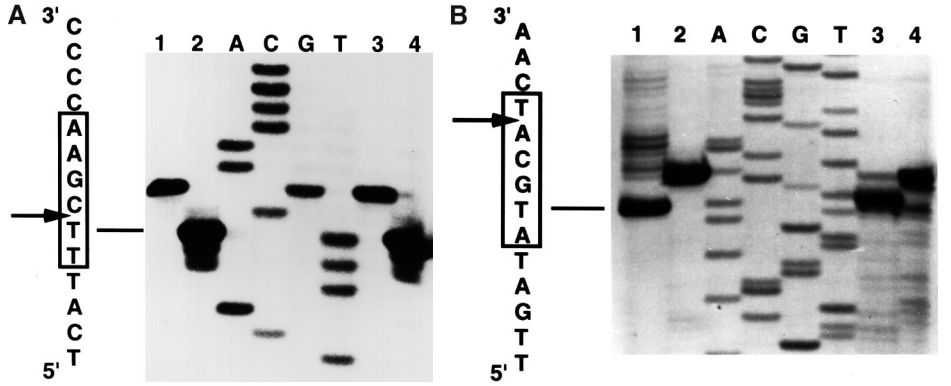

FIG. 4. Autoradiograms of a sequencing gel showing the recognition and cleavage sites for *Csp*68KII and *Csp*68KIII. (A) Autoradiogram of a sequencing gel showing the *Csp*68KII (*Asu*II) cleavage site. Lane 1: Klenow fill-in reaction of *Nsp*V-digested DNA, lane 2: *Nsp*V-digested DNA, lane 3: Klenow fill-in reaction of *Csp*68KII-digested DNA, lane 4: *Csp*68KII-digested DNA. The box surrounds the recognition site and the arrow indicates the position of cleavage. The same primer (KMNM5) was used for all of the above reactions. (B) Autoradiogram of a sequencing gel showing the *Csp*68KIII (*Ava*III) cleavage site. Lane 1: T4 polymerase reaction of *Ava*III-digested DNA, lane 2: *Ava*III-digested DNA, lane 3: T4 polymerase reaction of *Csp*68KIII-digested DNA, lane 4: *Csp*68KIII-digested DNA. Lanes A, C, G, and T are sequencing ladders generated by dideoxy sequencing of a region of pKMS3 which contain sites for *Csp*68KII, and *Csp*68KIII. The box surrounds the recognition site and the arrow indicates the position of cleavage.

cytosine and the internal guanine. These enzymes recognized identical sequences and cleaved the DNA at precisely the same location.

DISCUSSION

A combination of approaches including detection in crude lysates, and ion exchange chromatography has allowed us to identify the existence of additional type II restriction-modification systems in *Cyanothece* sp. strain BH68K. A simple two step purification using PII phosphocellulose (pH 7.0) and Mono Q FPLC columns in series led to the efficient separation of three restriction endonucleases: *Csp*68KI (*Ava*II), *Csp*68KII (*Asu*II), and *Csp*68KVI (*Fnu*DII). Restriction enzymes *Csp*68KII, *Csp*68KIII, and *Csp*68KVI restricted DNA at the recognition sites similar to those of *Asu*II (TTCGAA), *Ava*III (ATGCAT), and *Fnu*DII (CGCG), respectively, as shown by their cleavage patterns. Further verification that restriction occurs at these sites was demonstrated by primer extension analysis of labeled DNA restricted with *Cyanothece* sp. strain BH68K enzymes. Comparison of labeled DNA digested with commercially available enzymes showed an identical cleavage pattern. Therefore, the *Cyanothece* enzyme designated *Csp*68KII is an isoschizomer of *Asu*II and restricts DNA at 5′-TT/CGAA-3′. Cleavage occurs between the thymine and cytosine nucleotides, producing 2 bp 5′ overhang ends. *Asu*II is the prototype enzyme for this type of restriction, and was first identified in *Anabaena* sp. strain PCC 6309 [19]. Two examples of *Asu*II isoschizomers described in other species of cyanobacteria are *Nsp*V from *Nostoc* sp. strain PCC 7524 [20,21] and *Ssp*RFI from *Synechococcus* sp. strain RF-1 [22]. The *Cyanothece* enzyme designated *Csp*68KIII is an isoschizomer of *Ava*III and restricts DNA at 5′-ATGCA/T-3′, where cleavage occurs between the 3′ adenosine and thymine nucleotides producing four-base-pair 3′ overhang ends. *Ava*III is the prototype enzyme for this restriction, and was first reported in *Anabaena* sp. strain PCC 7118 [23]. The restriction activity of these enzymes was not detected during the characterization of *Csp*68KI [5] because the plasmids used in that analysis (pUC19 and pBR322) lack both *Csp*68KII (*Asu*II) and *Csp*68KIII (*Ava*III) sites. *Csp*68KVI is an isoschizomer of the restriction endonuclease, *Fnu*DII, isolated from the obligate anaerobe *Fusobacterium nucleatum* [24]. Restriction activity for *Csp*68KIV (*Hae*III) and *Csp*68KV (*Msp*I) were not detected

using the purification procedure employed in this study inspite of the presence of M.*Csp*68KIV and M. *Csp*68KV genes in *Cyanothece* sp. BH68K [7]. No corresponding restriction enzyme activity was detected in crude extract either. It is possible that these two methylases are present without their complementary restriction enzymes.

Some additional restriction enzyme activity was detected in phosphocellulose column (pH 7.0) wash-through collected prior to salt elution. These enzymes did not bind to the column under the conditions employed. Plasmid digestion using this column wash confirmed the presence of the restriction endonuclease, *Csp*68KIII (*Ava*III), as well as some other four-base-pair recognizing enzymes.

Cyanothece sp. strain BH68K has been shown to contain five HRM systems in addition to *Csp*68KI. These additional HRM systems have been designated *Csp*68KII, *Csp*68KIII, *Csp*68KIV, *Csp*68KV, and *Csp*68KVI. The enzymes *Csp*68KII *Csp*68KIII and *Csp*68KIV are isoschizomers of *Asu*II and *Ava*III, and *Fnu*DII respectively. Some species of cyanobacteria have been reported to have five HRM systems [20] and one organism is reported to have six [25]. In order to develop a functional system for gene transfer in *Cyanothece* sp., it is necessary to identify all restriction systems present so that vectors can be constructed that are devoid of pertinent restriction sites. Alternatively, the vectors can be methylated *in vitro* or *in vivo* prior to transfer.

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