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Reduction of Conjugal Transfer Efficiency by Three Restriction Activities of *Anabaena* sp. Strain PCC 7120

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The efficiency of conjugal transfer of plasmids from *Escherichia coli* to the cyanobacterium *Anabaena* sp. strain PCC 7120 was quantitated as a function of the number of restriction sites for the restriction enzymes carried by the recipient. In addition to the previously recognized isoschizomers of *Ava*I and *Ava*II, PCC 7120 was found to possess an isoschizomer of *Ava*III. Plasmids modified in *E. coli* with methylases that protect in vitro against restriction by the three enzymes were transferred with high efficiency, nearly independent of the number of restriction sites on the plasmid. Plasmids left unprotected against one of the three restriction enzymes were transferred with lower efficiencies. For low numbers of sites, the efficiency of conjugal transfer decreased as an exponential function of the number of unprotected sites. The methods presented may be used to increase the efficiency of conjugal transfer into restriction-competent bacteria.

Since first recognized, restriction in vivo has been most closely identified with the reduction of plating efficiency of bacteriophage (7, 10). Although conjugal transfer of unmodified DNA also was shown to be sensitive to restriction in *Escherichia coli* (10), the inability of certain restriction enzymes to act on single-stranded DNA (49, 56) has led to the view that conjugally transferred single-stranded DNA (37) should be immune from restriction (5, 69). Accordingly, there are many examples in which conjugal transfer is unaffected by restriction barriers that prevent the infection of unmodified phage or the transformation of unmodified DNA (5, 20, 36, 41, 55, 66). However, there are also reports of restriction in nonenteric bacteria indeed limiting conjugal efficiency (18, 44, 64, 79).

Regardless of the biological importance of restriction, the resulting inefficiency of DNA transfer is a frequently encountered barrier against introducing DNA into bacteria of experimental interest. While restriction may reduce the frequency of DNA transfer below levels of detectability (40, 42, 79), it is also possible that some introduced DNA with unmodified restriction sites may escape destruction. With this in mind, it is often of practical importance to know quantitatively the degree to which each restriction site impairs DNA transfer into restriction-competent recipients.

The effect of restriction sites on the efficiency of DNA transfer has been quantitated in several studies using plasmids or phage with a known number of sites. Such studies have measured the effect of restriction on phage infection (47, 48, 54), phage transfection (35), transformation (17, 51, 71), and electroporation (8, 34, 43). Reduction of conjugal transfer efficiency by restriction has not been systematically studied, although there have been some quantitative reports (30, 70).

Anabaena sp. strain PCC 7120 is a filamentous cyanobacterium capable of both nitrogen fixation, in specialized cells called heterocysts (78), and oxygenic photosynthesis. The strain possesses isoschizomers of both *Ava*I and *Ava*II [termed *Asp*(7120)I and *Asp*(7120)II, respectively] (19). The first successful attempt to introduce DNA into the strain was based in part on removal of most *Ava*I and *Ava*II sites from a shuttle vector capable of replication in both *Anabaena* sp. and *E. coli* (79). A more general means was later found to overcome restriction, by methylating plasmids in vivo prior to conjugation (23) or electroporation (67). This strategy has been used with other bacteria as well (14, 18, 42).

We report here the construction of plasmids carrying methylases that protect against one or more restriction activities of *Anabaena* sp. strain PCC 7120, including a third restriction activity reported here for the first time. Using these plasmids, we have systematically investigated how the number of restriction sites affects the efficiency of conjugal transfer.

MATERIALS AND METHODS

Strains and culture conditions. Strains of *Anabaena* sp. and *E. coli* used in this work are listed in Table 1. Wild-type *Anabaena* sp. strain PCC 7120 and a derivative (AV) deficient in *Ava*I restriction were grown at 30°C with continuous illumination from fluorescent lamps, on a rotary shaker in 125-ml Erlenmeyer flasks containing 50 ml of BG11 medium (58). Strain AMP2, a derivative of PCC 7120 that lacks sugar-nonspecific nuclease NucA, was grown in BG11 supplemented with 5 μg of neomycin sulfate (NM) per ml. Strains of *E. coli* carrying plasmids were grown in Luria broth supplemented, as appropriate, with 50 μg of ampicillin per ml, 25 μg of chloramphenicol per ml, 50 μg of kanamycin sulfate per ml, and/or 100 μg of spectinomycin dihydrochloride per ml.

Preparation of extracts from cells of *Anabaena* sp. Cells were harvested by centrifugation and stored at −20°C. Repeated freezing and thawing appeared necessary for release of *Asp*(7120)III activity, although it was not required for the release of the isoschizomers of *Ava*I and *Ava*II. After five cycles of thawing and freezing in liquid air, the pellet from a 50-ml culture was thawed and resuspended in 0.5 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride. The suspension was then frozen in liquid air in a prechilled mortar and ground. The homogenate was centrifuged at 10,000 × *g* for 15 min, and the resulting supernatant solution was used as crude extract. In certain cases, streptomycin sulfate was added to the crude extract of strain AMP2 to a final concentration of 1.5% in order to precipitate nucleic acids (33). The precipitate was removed by centrifugation at 10,000 × *g* for 20 min, and the resulting supernatant solution was desalted with Sephadex G-25 prior to use. Protein content was measured according to the method of Bradford (12). DNA was digested simultaneously by restriction enzymes present in the extract of

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Resistance[b]	Type[c]	Derivation or relevant characteristics	Source or reference(s)
Anabaena sp. strains			Wild type	R. Haselkorn
PCC 7120				
AMP2			*nucA*::Nm^r derivative, defective in extracellular nuclease	45
AV			Spontaneous mutant, defective in *Ava*I restriction	This study
E. coli K-12 strains				
DH1			*recA1* Nx^r	31
HB101			*recA13 mcrBC*	11
KA839			*mutS3*	27
SKA003			Derived from DH1; *recA1* Nx^r PM dependent	This study
Plasmids				
pACYC177	Ap^r Km^r		Source of $oriV_{p15A}$	15, 60
pBR322	Ap^r Tc^r		Source of $oriV_{pMB1}$ and $oriT_{pMB1}$	3
pCSAM49	Ap^r		Defined *Nsi*I site	This study
pDS4101	Ap^r	Helper	ColK::Tn*1*, Mob_{ColK}	72
pEco47IIM	Ap^r		Source of M.*Eco*47II (protects against R.*Ava*II)	A. Janulaitis, ESP Fermentas
pIC19H	Ap^r		Source of polylinker	39
pIC20H	Ap^r		Source of polylinker	39
pIC20R	Ap^r		Source of polylinker	39
pKL152$_{RM2-12}$	Ap^r		Source of M.*Ava*I (protects against R.*Ava*I)	K. D. Lunnen, New England BioLabs
pKT210	Cm^r Sm^r		Source of Cm^r	2
pRL5	Cm^r Sm^r		Parent of pRL1285 and pRL1286	79
pRL25	Km^r	Cargo	0 *Ava*I, 1 *Ava*II, 0 *Ava*III sites	75
pRL25C	Km^r	Cargo	0 *Ava*I, 2 *Ava*II, 0 *Ava*III sites	75
pRL35RL	Km^r	Cargo	0 *Ava*I, 3 *Ava*II, 1 *Ava*III sites	This study
pRL139	Ap^r		Source of inverted polylinker	28
pRL171	Ap^r Cm^r		Positive selection vector	24
pRL443	Ap^r Tc^r	Conjugal	Km^s derivative of RP4	This study
pRL444	Km^r	Cargo	1 *Ava*I, 3 *Ava*II, 0 *Ava*III sites	21
pRL447	Km^r		Source of polylinker	24
pRL488	Km^r	Cargo	2 *Ava*I, 3 *Ava*II, 0 *Ava*III sites	21, 25
pRL518	Cm^r	Helper	Mob_{ColK}, M.*Eco*47II	This study
pRL528	Cm^r	Helper	Mob_{ColK}, M.*Ava*I, M.*Eco*47II	This study
pRL530	Cm^r	Helper	Mob_{ColK}, M.*Ava*I	This study
pRL539	Km^r	Cargo	0 *Ava*I, 1 *Ava*II, 0 *Ava*III sites	This study
pRL540	Km^r	Cargo	0 *Ava*I, 2 *Ava*II, 0 *Ava*III sites	This study
pRL541	Km^r	Cargo	3 *Ava*I, 3 *Ava*II, 0 *Ava*III sites	This study
pRL542	Cm^r	Helper	Mob_{ColK}	This study
pRL545	Km^r	Cargo	0 *Ava*I, 3 *Ava*II, 0 *Ava*III sites	This study
pRL546	Km^r	Cargo	1 *Ava*I, 3 *Ava*II, 0 *Ava*III sites	This study
pRL548	Km^r	Cargo	1 *Ava*I, 1 *Ava*II, 0 *Ava*III sites	This study
pRL554	Km^r	Cargo	1 *Ava*I, 9 *Ava*II, 0 *Ava*III sites	This study
pRL591-W45	Km^r		Source of M.*Eco*T22I (protects against R.*Ava*III)	22
pRL623	Cm^r	Helper	Mob_{ColK}, M.*Ava*I, M.*Eco*47II, M.*Eco*T22I	This study
pRL1045	Km^r	Helper	Mob_{ColK}, M.*Ava*I, M.*Eco*47II	This study
pRL1049	Sm^r Sp^r	Cargo	0 *Ava*I, 1 *Ava*II, 0 *Ava*III sites	9
pRL1050	Sm^r Sp^r	Cargo	2 *Ava*I, 3 *Ava*II, 0 *Ava*III sites	77
pRL1095a	Km^r Sm^r Sp^r	Cargo	4 *Ava*I, 4 *Ava*II, 2 *Ava*III sites	This study
pRL1095aa	Km^r Sm^r Sp^r	Cargo	6 *Ava*I, 5 *Ava*II, 4 *Ava*III sites	This study
pRL1096a	Km^r Sm^r Sp^r	Cargo	2 *Ava*I, 2 *Ava*II, 2 *Ava*III sites	This study
pRL1097b	Km^r Sm^r Sp^r	Cargo	3 *Ava*I, 4 *Ava*II, 1 *Ava*III sites	This study
pRL1108a	Km^r Sm^r Sp^r	Cargo	5 *Ava*I, 5 *Ava*II, 3 *Ava*III sites	This study
pRL1123a	Cm^r	Helper	Mob_{ColK}, M.*Eco*47II, M.*Eco*T22I	This study
pRL1124	Km^r	Helper	M.*Ava*I, M.*Eco*47II, M.*Eco*T22I	This study
pRL1132a	Cm^r	Helper	Mob_{ColK}, M.*Ava*I, M.*Eco*T22I	This study
pRL1277a	Ap^r Km^r	Cargo	pRL139(*Bam*HI)::cassette C.K1 (24)	This study
pRL1282	Km^r Sm^r Sp^r	Cargo	0 *Ava*I, 2 *Ava*II, 0 *Ava*III sites	This study
pRL1283	Km^r Sm^r Sp^r	Cargo	2 *Ava*I, 2 *Ava*II, 0 *Ava*III sites	This study
pRL1284	Km^r Sm^r Sp^r	Cargo	4 *Ava*I, 4 *Ava*II, 0 *Ava*III sites	This study
pRL1285	Km^r Sm^r	Cargo	1 *Ava*I, 3 *Ava*II, 0 *Ava*III sites	This study
pRL1286	Km^r Sm^r	Cargo	3 *Ava*I, 3 *Ava*II, 0 *Ava*III sites	This study
pTB7	Ap^r Tc^r		Source of *luxAB* from *Vibrio harveyi*	4
pUC4K	Ap^r Km^r		Source of Km^r cassette	74
RP4	Ap^r Km^r Tc^r	Conjugal	IncP conjugal plasmid	68

[a] Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Mob, mobilization; Nm, neomycin; Nx, nalidixic acid; *oriV*, origin of replication; *oriT*, origin of transfer; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

[b] Km^r determinants also confer resistance to NM.

[c] See text and reference 23 for explanation of the terms conjugal, helper, and cargo.

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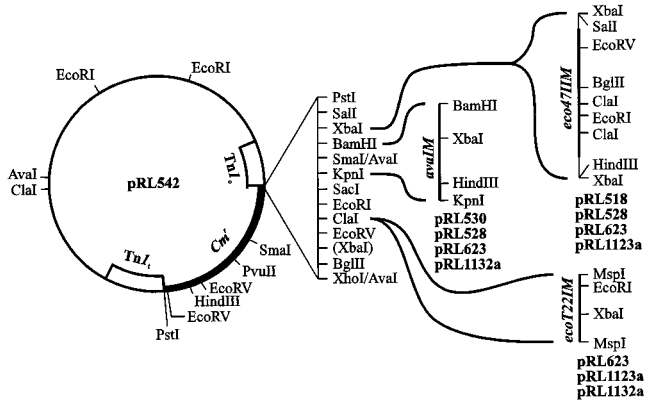

FIG. 1. Cm^r helper plasmids. All the plasmids shown are variants of pRL542, carrying a ColK replicon and *mob* genes from ColK that permit mobilization of pBR322 derivatives by some conjugal plasmids. The remaining Cm^r helper plasmids were obtained by insertion of methylase gene-containing cassettes into the polylinker in various combinations, except that pRL1123a was made as a product of partial *Hin*dIII digestion of pRL623 and pRL1132a was made from pRL623 by replacing *eco47IIM* from *Bam*HI to the distal *Sal*I site with a fragment from pUC4K. Plasmids carrying each cassette are indicated. All restriction sites are shown for *Cla*I, *Eco*RI, *Hin*dIII, *Pst*I, and *Sal*I. pRL542 consists of pDS4101 (ColK::Tn*1*) with a Cm^r determinant linked to a polylinker placed between the two outer *Pst*I sites of Tn*1*, one on the transposase proximal side (Tn1_t) and the other on the other side (Tn1_o). The Cm^r determinant was taken from pKT210 as a 2.7-kb *Pst*I-*Xho*I fragment (the gene is probably oriented *Pst*I to *Xho*I). The *Xho*I site was fused to the *Xho*I-*Pst*I polylinker taken from pIC20H. All known sites are shown for restriction enzymes that cut within the polylinker. The *Xba*I site in parentheses can be cut only when the plasmid is isolated from a *dam* mutant strain. To construct the M.*Ava*I cassette, *avaIM* was inserted as a 1.7-kb *Stu*I-*Xmn*I fragment from pKL152$_{RM2\text{-}12}$ into the *Sma*I site of the pIC20H polylinker and extracted by cutting at the adjacent *Bam*HI and *Kpn*I sites. The internal *Hin*dIII site is on the side of the fragment closer to the gene encoding R.*Ava*I on pKL152$_{RM2\text{-}12}$ (data not shown). To construct the M.*Eco*47II cassette, *eco47IIM* was inserted as a 3.5-kb *Hin*dIII-*Sal*I fragment from pEco47IIM between the *Hin*dIII and *Sal*I sites of the polylinker of pRL447 and extracted by cutting at the adjacent *Xba*I sites. The cassette contains pBR322 sequences from *Hin*dIII to *Bam*HI on one side of *eco47IIM* and from *Bam*HI to *Sal*I on the other. The orientation of the gene is unknown. To construct the M.*Eco*T22I cassette, *ecoT22IM* was inserted as a 1.1-kb *Msp*I fragment from pRL591-W45 into the *Cla*I site of the pIC20H polylinker. The internal *Eco*RI site is on the side of the fragment closer to the gene encoding R.*Eco*T22I on pRL591-45.

Anabaena sp. for 2 to 4 h at 37°C, in a buffer consisting of 100 mM NaCl, 10 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.5).

Determination of sites of cleavage by *Asp*(7120)III. Isolated fragments obtained by digestion of pCSAM49 with an extract of AMP2 were denatured and annealed to the universal or reverse sequencing primers directed against pUC plasmids. The primers were extended with T7 DNA polymerase (Pharmacia), labeling was introduced with α-^{35}S-thio-dATP, and the products of the reactions were subjected to electrophoresis in urea-polyacrylamide gels (61). Corresponding sequencing ladders obtained by dideoxy chain termination (63) used the same primers and plasmid pCSAM49 as template.

Construction of plasmids. A family of Cm^r helper plasmids (23) based on pDS4101 and encoding Mob proteins from ColK is depicted in Fig. 1. The plasmids are able to mobilize plasmids carrying *oriT* of pMB1 (e.g., derivatives of pBR322). All but pRL542 carry genes that encode one or more of the methylases M.*Ava*I, M.*Eco*47II, and M.*Eco*T22I. Km^r helper plasmids pRL1045 and pRL1124 were based on pACYC177, a plasmid compatible with pBR322 derivatives. These helpers lack any *mob* genes and are therefore used to methylate cargo plasmids (23) with origins of transfers recognized by the Mob products encoded by the conjugal plasmid itself (76). pRL1045 and pRL1124 consist of a *Pvu*II-*Pst*I fragment of pRL528 or pRL623, respectively, between the *Sca*I and *Pst*I sites of pACYC177. Helper plasmids carrying genes encoding M.*Ava*I or M.*Eco*47II could not be propagated in *E. coli* strains that are *mcrBC*$^+$, owing to the ability of the McrBC endonuclease to cut certain cytosine-methylated DNA (52), so HB101, lacking McrBC function (53), was used as the host strain.

Cargo plasmids (23) termed *Ava*I series 1 (pRL545, pRL540, pRL444, pRL546, pRL488, and pRL541) were designed to be minimally different except for the number of *Ava*I sites, in a plasmid capable of replication in *Anabaena* sp. strain PCC 7120. All the plasmids are similar to pRL488 (12.3 kb), which contains a cyanobacterial replicon from pDU1, the origins of transfer and replication from pBR322, and genes encoding luciferase (*Vibrio fischeri luxAB*) and neomycin phosphotransferase (*npt*). pRL444 (12.3 kb) differs from pRL488 only by the presence of an additional *Ava*I/*Sma*I site in its polylinker. An *Ava*I site was removed from pRL444 by filling in its *Xho*I site to make pRL545 (12.3 kb) or by deleting the *Xho*I-*Sal*I fragment within pRL444 to make pRL540 (11.6 kb). An *Ava*I site was removed from pRL488 by filling in its *Xho*I site to make pRL546 (12.3 kb). Two additional *Ava*I sites were added to pRL444 to create pRL541 (12.3 kb) by replacing the *Sal*I-*Sst*I portion of the polylinker of pRL444 with a *Sal*I-*Sst*I polylinker region from pIC19H. For this series, methylation was provided by pRL518 (test) or pRL528 (control).

Cargo plasmids termed *Ava*I series 2 (pRL1282, pRL1285, pRL1283, pRL1286, and pRL1284) all carry the pDU1 replicon for replication in *Anabaena* sp. strain PCC 7120. The plasmids were based on pRL1050, a derivative of pRL25 carrying the Sm^r Sp^r determinant from Tn*7* and *luxAB* from *V. fischeri*; pRL1049, the immediate precursor of pRL1050 lacking *luxAB*; or pRL5, a relative of pRL25 carrying the Sm^r determinant from R300B and Cm^r from pBR325. pRL1282 (11.3 kb) differs from pRL1049 only by the addition of a Km^r determinant: the 1.3-kb cassette C.K1 (24) from pRL1277a was inserted into the unique *Bam*HI site of pRL1049, providing an additional *Ava*II site. A similar plasmid, pRL1283 (11.3 kb), contains two additional *Ava*I sites, because the C.K1 Km^r cassette, flanked by *Eco*RI-*Bam*HI polylinkers (L.EHE1 [24]), each containing an *Ava*I site, was inserted into the unique *Eco*RI site of pRL1049. pRL1284 (13.7 kb) was formed by placing the same *Eco*RI-bounded Km^r cassette into the corresponding *Eco*RI site of pRL1050. pRL1285 and pRL1286 (both 12.4 kb) were derived from pRL5 by placing C.K1 flanked by L.EHE1 digested with *Sma*I or *Eco*ICRI, respectively, in the unique *Fsp*I site of pRL5. The polylinker retained two *Ava*I sites in the latter but not the former case. For this series, methylation was provided by pRL1123a (test) or pRL623 (control) or, alternatively, by pRL518 (test) or pRL528 (control).

Cargo plasmids termed *Ava*II series 1 (pRL548, pRL25, pRL539, pRL25C, pRL540, pRL545, pRL546, and pRL554) were designed to be minimally different except for the number of *Ava*II sites, in a plasmid capable of replication in *Anabaena* sp. strain PCC 7120. All the plasmids are similar to pRL488. pRL25 (9.9 kb) differs from pRL488 in the absence of a polylinker, a 269-bp noncoding region, and *luxAB* and the presence of a 378-bp fragment derived from pBR322. pRL25C (10.2 kb) differs from pRL25 by the presence of the *cos* site from bacteriophage lambda. Two *Ava*II sites and one *Ava*I site were removed from pRL444 by deleting its 2,427-bp *Bam*HI-*Bam*HI fragment, to form pRL539 (9.8 kb). Two *Ava*II sites and one *Ava*I site were removed from pRL488 by replacing its 2,439-bp *Bam*HI-*Sal*I fragment with the *Sal*I-*Bgl*II portion of the polylinker of pIC20R, to form pRL548 (9.8 kb). Six *Ava*II sites were added to pRL488, at the expense of an *Ava*I site, by inserting the 1,415-bp *Sal*I-*Pvu*II fragment from pBR322 between the *Sal*I and *Sma*I sites of pRL488, to form pRL554 (13.7 kb). The construction of plasmids pRL540, pRL545, and pRL546 is described above. For this series, methylation was provided by pRL530 (test) or pRL528 (control).

Cargo plasmids termed *Ava*II series 2 (pRL25, pRL1096a, pRL35RL, pRL1095a, and pRL1095aa) were all based on pRL25 or its derivative pRL1049 or pRL1050. pRL1096a (11.2 kb) differs from pRL1049 by the addition of the Km^r determinant from pUC4K (containing two *Ava*I, one *Ava*II, and two *Ava*III sites), excised with *Eco*RI and placed in the unique *Eco*RI site of pRL1049. pRL35RL (12.3 kb) differs from pRL25 by the insertion of *luxAB* from *Vibrio harveyi* (containing zero *Ava*I, two *Ava*II, and one *Ava*III sites) into the *Bam*HI site of pRL25. *luxAB* was taken as a *Bst*XI-*Pvu*II fragment from pTB7 and supplied with *Bam*HI ends by cloning it (after blunting with S1 nuclease) into the *Sma*I site of pRL171. pRL1095a (13.6 kb) and pRL1095aa (14.8 kb) were obtained by inserting one or two copies, respectively, of the Km^r determinant from pUC4K, excised with *Eco*RI, into the unique *Eco*RI site of pRL1050. For this series, methylation was provided by pRL1132a (test) or pRL623 (control).

Cargo plasmids termed the *Ava*III series (pRL25, pRL35RL, pRL1095a, pRL1108a, and pRL1095aa) were all based on pRL25 or its derivative pRL1050. pRL1108a (14.5 kb) differs from pRL1050 by the addition of one full and one partial copy of the Km^r determinant from pUC4K. One copy was inserted into the unique *Eco*RI site of pRL1050, with an orientation opposite to that in pRL1095a. The internal *Nsi*I fragment was removed from that copy (thereby reducing by one the number of *Ava*III sites), and a second, complete copy, excised with *Hin*cII, was inserted into the unique *Nru*I site within the first Km^r determinant (in opposite orientation), yielding pRL1108a. The construction of plasmids pRL35RL, pRL1095a, and pRL1095aa is described above. For this series, methylation was provided by pRL528 (test) or pRL623 (control).

pRL443, a Km^s derivative of conjugal plasmid RP4, was constructed according to the method of Slutzky et al. (65). A paromomycin (PM)-dependent derivative of *E. coli* DH1, SKA003, was isolated by plating the strain on 200 μg of PM per ml and propagating it in Luria broth plus 100 μg of PM per ml. RP4 was transferred into the *E. coli* mutator strain KA839 and from there transferred into SKA003. Selection was applied with nalidixic acid (20 μg/ml) against KA839, with tetracycline (15 μg/ml) for the presence of RP4, and with PM (100 μg/ml) to meet the requirement of PM dependence. A plasmid from one of several colonies that appeared was recovered by mating it to a Pm^s strain and called pRL443. pRL443 reverted to Km^r with a frequency of about 3×10^{-9}, consistent with the lesion being a point mutation.

pCSAM49 was constructed by inserting the 185-bp *Cla*I-*Sma*I fragment from the *npt* gene of pUC4K between the *Cla*I site and the S1-blunted *Kpn*I site of pIC20R. The *Ava*I site regenerated by this construction was destroyed by filling

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it in with Klenow fragment. The insertion places a single *Ava*III site 156 bp from the *Cla*I site within the polylinker.

Conjugal transfer of plasmids from *E. coli* to *Anabaena* sp. strain PCC 7120. The plate mating procedure of Elhai and Wolk (23) was modified so as to obviate the need for the large number of expensive filters otherwise required and to reduce the number of conjugal events from two to one, eliminating one possible source of variation. Prior to the day of the experiment, an exponentially growing culture of *Anabaena* was fragmented to an average of two to three cells per filament by cavitation, normally for a total of 10 min, in a bath sonicator (see reference 23). The progress of fragmentation was monitored by microscopy. Fragmented filaments were pelleted (for the *Ava*I and *Ava*II series 2 and the *Ava*III series, also repeatedly washed), resuspended in fresh medium, and allowed to recover overnight under growth conditions. The next day, they were harvested by centrifugation at about 1,200 × *g* and resuspended in a volume equal to approximately twice the volume of the pellet. The concentration of filaments in the resulting suspension was determined with a hemocytometer.

Three-milliliter overnight cultures of *E. coli* HB101 carrying three plasmids—the conjugal plasmid pRL443, a helper plasmid, and a cargo plasmid—were diluted 1:20 and grown for approximately 2 h to an optical density at 600 nm of about 0.5. For each mating, a 1.5-ml volume of each *E. coli* culture was washed once with 1 ml of Luria broth and then centrifuged in a microcentrifuge tube. Aliquots of cyanobacterial suspension containing about 3×10^7 short filaments derived from fragmentation were added to each tube of sedimented *E. coli* cells and mixed by gentle pipetting.

The resulting mating mixtures were transferred to petri dishes containing BG11 plus 1% Difco agar, purified as described elsewhere (13), and for the *Ava*I and *Ava*II series 2 and the *Ava*III series supplemented with 5% (vol/vol) Luria broth. The mixtures were allowed to dry on the plate and then incubated under *Anabaena* sp. growth conditions for 24 h. The mixtures were washed from the surface of the agar plates with a total of 1 ml of BG11 medium and pipetted into microcentrifuge tubes. The resulting suspensions were serially diluted, and 100 and/or 300 μl of appropriate dilutions was spread with glass beads on plates of BG11 supplemented with 1% Difco agar (purified as described elsewhere [13]), with or without 25 or 40 μg of NM per ml. The plates were incubated under growth conditions for up to 3 weeks, and colonies were counted.

The effect of restriction on conjugal transfer was estimated with a parameter, *F*, that was calculated, upon transfer of a test plasmid from any particular experimental series into *Anabaena* sp., as the ratio of the number of neomycin-resistant colonies obtained with that test plasmid to the number of neomycin-resistant colonies obtained with the fully modified control plasmid from that experimental series (i.e., *Ava*I, *Ava*II, or *Ava*III). Test plasmids were modified with a helper plasmid (identified above as "test") that methylated the target sites for all of the three restriction-modification systems (*Ava*I, *Ava*II, and *Ava*III) except the system that was under consideration.

The absolute efficiency of conjugal transfer of fully methylated plasmids ranged from experiment to experiment from 0.01 to 0.1 Nm^r colonies per CFU without selection. Efficiencies measured in this way are higher with unfragmented filaments than with filaments fragmented by cavitation because, in the former case, the CFU, the single filament, typically averages hundreds of cells rather than the several cells in the overnight cultures of fragmented filaments such as those used in these experiments.

RESULTS

A third restriction endonuclease in *Anabaena* sp. strain PCC 7120. Lambda DNA was digested by an extract of strain PCC 7120 to determine whether the strain possesses any restriction activity beyond the two already reported. The resulting pattern (Fig. 2A) was inconsistent with the presence of only *Asp*(7120)I and *Asp*(7120)II, isoschizomers of *Ava*I and *Ava*II, respectively (Fig. 2B). Since *Anabaena* sp. strain PCC 7118, a strain similar to *Anabaena* sp. strain PCC 7120 (57), possesses *Ava*III (59) in addition to *Ava*I and *Ava*II (46), we compared the pattern in Fig. 2A with the pattern resulting from digestion of lambda DNA with *Ava*I, *Ava*II, and *Nsi*I, an isoschizomer of *Ava*III (Fig. 2B). The two patterns appeared nearly identical.

To provide a different test of the identity of the additional restriction activity, extract from PCC 7120 was used to digest a plasmid, pCSAM49, constructed to have a single *Nsi*I site, two *Ava*II sites, and no *Ava*I site. The resulting fragments comigrated with those resulting from digestion with authentic *Nsi*I and *Ava*II (Fig. 2C), superimposed on fragments resulting from digestion with *Ava*II alone. The fragment sizes are consistent with the interpretation that PCC 7120 possesses a third endonuclease, termed *Asp*(7120)III, that digested pCSAM49 at the same site as *Nsi*I (but under the experimental conditions

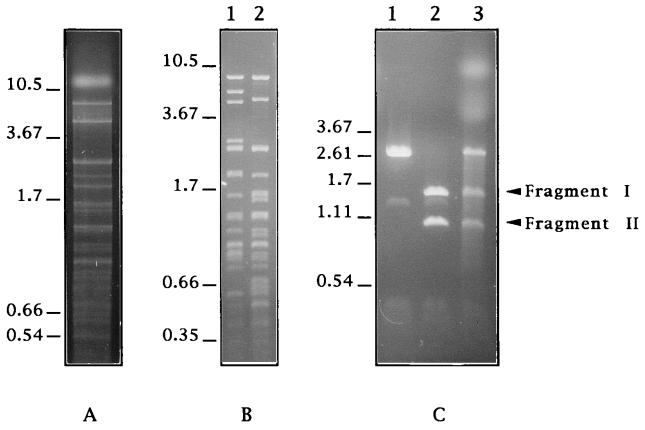

FIG. 2. Restriction pattern of coliphage λ or plasmid pCSAM49 digested with extracts of *Anabaena* sp. strain PCC 7120 or with commercially obtained *Ava*I, *Ava*II, and *Nsi*I. The sizes of standards are indicated (in kilobases). (A) λ DNA digested with extract of *Anabaena* sp. strain PCC 7120 containing 40 μg of protein. A small quantity of DNA was used, to enhance digestion. (B) λ DNA digested with commercially obtained *Ava*I and *Ava*II (lane 1) or *Ava*I, *Ava*II, and *Nsi*I (lane 2). The sizes of the largest fragments are predicted to be 7,717, 5,644, and 4,716 bp (lane 1) and 7,717 and 4,967 bp (lane 2). (C) Plasmid pCSAM49 digested with *Ava*I and *Ava*II (lane 1); *Ava*I, *Ava*II, and *Nsi*I (lane 2); or streptomycin sulfate-treated extract from derivative AMP2 of *Anabaena* sp. strain PCC 7120 containing 188 μg of protein (lane 3). The single *Nsi*I site of pCSAM49 divides the larger *Ava*II fragment (2,612 bp) into the two indicated fragments (1,549 and 1,063 bp).

used, only partially). It is likely that *Asp*(7120)III has the same specificity, 5′-ATGCAT-3′, as *Nsi*I and *Ava*III and not a degenerate specificity, since the largest band in the *Ava*I-plus-*Ava*II digest of lambda DNA (Fig. 2B, bp 39479 to 47605) (62) contains every possible hexameric sequence differing from ATGCAT by a single nucleotide and yet is not cut by the extract.

The cleavage site of *Asp*(7120)III was determined by primer extension of the two fragments (fragments I and II in Fig. 2C) of pCSAM49 cut with the extract and deduced to contain ends produced by the novel restriction activity. Primer extension should yield fragments whose last nucleotides abut the cut site on the template strand. Figure 3 shows that these nucleotides are the 5′ A of 5′-ATGCAT-3′ and the A pairing with the 3′ T, as predicted from cleavage as follows:

5′-A T G C A|T-3′
3′-T|A C G T A-5′

Helper plasmids with genes encoding methylases that protect against restriction by enzymes of *Anabaena* sp. strain PCC 7120. In order to assess the degree to which restriction affects the efficiency of conjugal transfer of plasmid DNA from *E. coli* to *Anabaena* sp., plasmids were constructed with different numbers of sites for *Ava*I, *Ava*II, and *Ava*III. All these cargo plasmids (listed in Table 1) were ultimately derived from pRL1 (79), a shuttle vector capable of replication in *Anabaena* sp. by virtue of a cyanobacterial origin of replication from pDU1 and capable of replication in *E. coli* by virtue of the origin of replication from pMB1, through pBR322. These plasmids also carry the origin of transfer (*oriT*) of pMB1.

Plasmids carrying *oriT* from pMB1 (related to ColE1) require the presence of *mob* gene products to be mobilized by conjugal plasmids. The *mob* gene of pMB1 itself is sufficient, but those of ColD and ColK will serve as well (26). We constructed a family of plasmids based on ColK, carrying its *mob* gene and, optionally, one or more of three methylases

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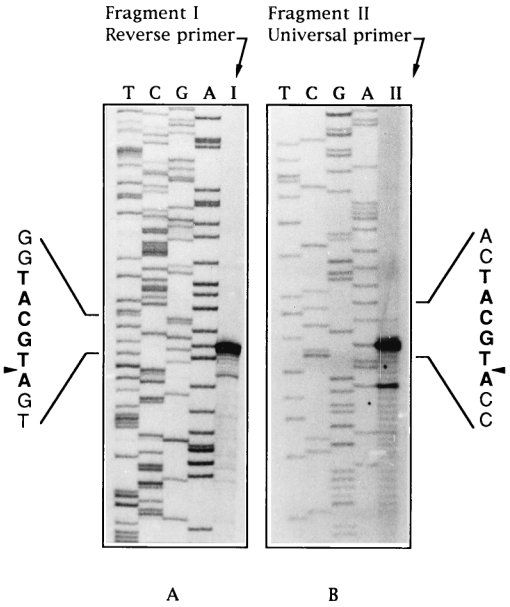

FIG. 3. Determination of sites of cleavage by *Asp*(7120)III. The sequence targeted by *Asp*(7120)III is indicated in boldface. Arrowheads indicate the deduced cleavage sites. Fragments I (A) and II (B) (Fig. 2C) obtained by digestion of pCSAM49 with an extract from derivative AMP2 of *Anabaena* sp. strain PCC 7120 were denatured and annealed to the reverse or universal pUC plasmid sequencing primer, respectively, which was extended. Corresponding sequencing ladders with plasmid pCSAM49 as template are shown.

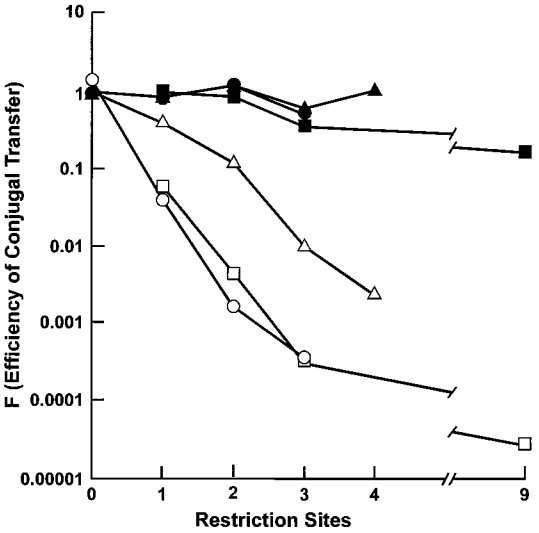

FIG. 4. Efficiencies of conjugal transfer of plasmids with different numbers of sites for *Ava*I, *Ava*II, and *Ava*III. Plasmids were transferred from *E. coli* to *Anabaena* sp. strain PCC 7120 (*Ava*I series 1 and *Ava*II series 1) and its derivative, AV (*Ava*III series), as described in Materials and Methods. The parameter *F* represents the ratio of neomycin-resistant colonies obtained under the experimental conditions to those obtained under conditions approximating no *Asp* (7120) restriction (see Materials and Methods). Each point represents an average of values from one to three experiments, with the most extreme values deviating from the mean by (on average) 29%. Each plasmid of *Ava*I series 1 and of *Ava*II series 1 was used at least once. Plasmids of *Ava*I series 1 were modified against *Ava*II by pRL518 (○) or against *Ava*I and *Ava*II by pRL528 (●). Plasmids of *Ava*II series 1 were modified against *Ava*I by pRL530 (□) or against *Ava*I and *Ava*II by pRL528 (■). Plasmids of *Ava*III series were modified against *Ava*I and *Ava*II by pRL528 (△) and against *Ava*I, *Ava*II, and *Ava*III by pRL623 (▲).

that protect against restriction by the restriction enzymes of *Anabaena* sp. strain PCC 7120. Some of these plasmids, called helper plasmids (23) because they facilitate conjugal transfer of plasmids, are described further in Fig. 1. The ColK-derived helper plasmids are quite stable, even without selection, enabling them to be used with plasmids carrying the same antibiotic resistance marker.

Effect of prior methylation on efficiency of conjugal transfer. Since removing known *Ava*I and *Ava*II sites increased the efficiency of conjugal transfer of plasmids into *Anabaena* sp. strain M-131 (79), we anticipated that protecting against the endonucleases by in vivo methylation ought to do the same. Series of shuttle plasmids, similar to each other except in the number of *Ava*I, *Ava*II, or *Ava*III sites, were constructed and used to assess the degree to which an unprotected restriction site diminishes the efficiency of conjugal transfer from *E. coli* to *Anabaena* sp.

In order to compare the effects on conjugal transfer of one restriction activity relative to another, it was necessary to normalize transfer efficiency to a common standard. We chose to normalize relative to the condition that we supposed would most closely approximate the absence of any effect by restriction: conjugal transfer of the same plasmid, fully methylated against *Ava*I, *Ava*II, and *Ava*III sites that it contained. For this normalization to be valid, we needed to determine whether prior methylation of restriction sites in *E. coli* effectively protected plasmids against the corresponding restriction enzyme of *Anabaena* sp.

Helper plasmid pRL528, encoding methylases that target the same sites as *Asp*(7120)I and *Asp*(7120)II, was adequate to protect almost completely against restriction by both. In its presence, conjugal transfer efficiency dropped only 2-fold from zero to three *Ava*I restriction sites, while dropping almost 3,000-fold when *Ava*I sites were not protected by methylation, and dropped only 6-fold from one to nine *Ava*II restriction sites, while dropping almost 40,000-fold when *Ava*II sites were not protected (Fig. 4). Helper plasmid pRL623, encoding methylases that target all of the restriction sites targeted by *Asp*(7120)I, *Asp*(7120)II, and *Asp*(7120)III, protected against loss of conjugal transfer efficiency resulting from all of those enzymes. For example, there was no significant difference in the efficiency of conjugal transfer when pRL623 was used to mobilize plasmids with as few as one *Ava*II site or as many as six *Ava*I, five *Ava*II and four *Ava*III sites (pRL25 versus pRL1095aa [Fig. 4]). Both pRL528 and pRL623 protected against digestion in vitro by *Ava*I, *Ava*II, and, in the case of pRL623, *Ava*III (data not shown).

Figures 4 and 5 show for *Ava*I, *Ava*II, and *Ava*III that each additional restriction site within a cargo plasmid, up to a point, diminished its efficiency of conjugal transfer. The fact that the log of conjugal transfer efficiency decreased initially in proportion to the number of restriction sites (above the lowest number in a series) indicates that each site diminished efficiency by a constant fraction: roughly 30-fold, 15-fold, and 5-fold in trials for *Ava*I, *Ava*II, and *Ava*III, respectively. In some trials, as in the *Ava*II series illustrated in Fig. 4, a point was reached beyond which additional restriction sites in the cargo plasmids had little effect on the efficiency of conjugal transfer.

Characterization of a spontaneous mutant deficient in *Ava*I restriction. In the course of these experiments, we identified a spontaneous derivative of *Anabaena* sp. strain PCC 7120 that readily accepted plasmids with up to four unmethylated *Ava*I sites (Fig. 5). Conjugal transfer efficiencies were still reduced by unprotected *Ava*III (Fig. 4) or *Ava*II sites (*Ava*II series 2 [data not shown]). Once a strain (called AV) was isolated through several successive single colony isolations and regrown, extracts were found to lack measurable *Ava*I activity in

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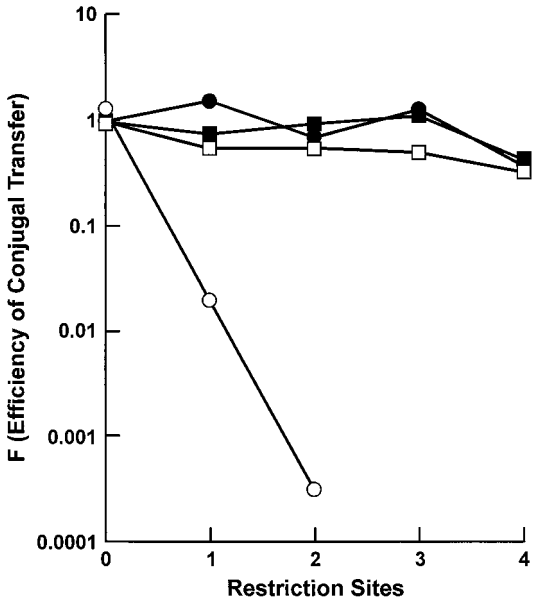

FIG. 5. Efficiencies of conjugal transfer to *Anabaena* sp. (○ and ●, wild-type PCC 7120; □ and ■, derivative AV), as measured by the parameter *F*, of plasmids with different numbers of sites for *Ava*I (series 2) in cargo plasmids. Plasmids were modified against *Ava*II and *Ava*III by pRL1123a (open symbols) and against *Ava*I, *Ava*II, and *Ava*III by pRL623 (solid symbols). Upon attempted transfer to wild-type PCC 7120 of pRL1123a-modified plasmids with three or four sites for *Ava*I, no neomycin-resistant exconjugants were obtained.

vitro (Fig. 6), although *Ava*II activity was normal (data not shown). DNA extracted from PCC 7120 and from AV was digested with *Ase*I or *Ssp*I and probed with a plasmid bearing *avaIMR* from *Anabaena variabilis*. The plasmid, pKL152RM2-12 (insert size, ca. 5.5 kb), hybridized to fragments that summed to ca. 7 and 8 kb for *Ase*I and *Ssp*I, respectively, but the labeled fragments showed no restriction fragment length polymorphisms between the two strains (data not shown). Therefore, no sizable insertion sequence had entered into the region of the PCC 7120 chromosome that hybridized with the *avaIMR*-containing clone from *A. variabilis.*

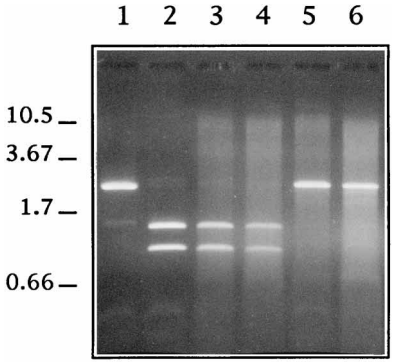

FIG. 6. Absence of *Ava*I activity in *Anabaena* sp. strain AV. *Ava*II-digested pIC19R (lane 1) was incubated in the presence of *Ava*I (lane 2), of extract from *Anabaena* sp. strain PCC 7120 (lanes 3 and 4), or of extract from strain AV (lanes 5 and 6). The extract used contained either 12.8 (lanes 3 and 5) or 32 (lanes 4 and 6) μg of protein. The sizes of standards are indicated (in kilobases).

DISCUSSION

Restriction prevents the transfer of DNA into a large number of laboratory bacteria of physiological interest, including *Anabaena* sp. strain PCC 7120, an organism that is important in the study of developmentally regulated nitrogen fixation and pattern formation (78). We have demonstrated here the ability of helper plasmids carrying genes encoding specific methylases to overcome restriction during conjugal transfer of DNA into *Anabaena* sp. The high efficiencies of conjugal transfer resulting from their use permit the formation of libraries of transposon-generated mutants (76) and the isolation of rare exconjugants in which plasmids have been integrated into the chromosome by homologous recombination (25, 29).

So long as the efficiency of conjugal transfer exceeds 0.1%, each of the restriction activities from *Anabaena* sp. strain PCC 7120 acts to diminish the efficiency of conjugal transfer by a factor that is more or less constant for each additional restriction site. For a large number of sites, the logarithmic relationship breaks down and conjugal transfer efficiency appears relatively insensitive to the number of sites. A similar phenomenon has long been known regarding the restriction of coliphage λ, for which increasing the number of *Eco*K sites from zero to three progressively decreases the efficiency of infection but sites beyond four have little additional effect (47). To explain the inability of a single restriction system to diminish infection beyond a certain level, Arber and Linn (1) argued for the existence of a rare physiological state in which restriction is not a factor, for example, owing to the rate of mutation to a restriction-negative phenotype. Alternatively, the plateau in conjugal transfer efficiency may be due to a limitation on all cells, for example, the titration of restriction enzymes by a large number of restriction sites (10).

The degree to which we found that restriction diminishes conjugal transfer falls within the range observed with other modes of DNA transfer. Infection of *E. coli* by λ was diminished 10- to 50-fold per *Eco*K site (47) and 3- to 10-fold per *Eco*RI site, depending on the specific site (48, 54). Transfection of *E. coli* by phage fd DNA was reduced 30- to 50-fold per *Eco*B site (35). There are fragmentary reports on restriction sites and the efficiency of transformation or electroporation of plasmid DNA that fall within the same range (8, 17, 71) or somewhat outside it (34, 51). Conjugal transfer appears similarly subject to restriction, at least in two reported cases. Trieu-Cuot et al. (70) found a 3,000-fold drop in conjugal transfer to *Bacillus subtilis*, owing to three unmodified *Bsu*MI sites and corresponding to about 15-fold per site. Guiney (30) tested the efficiency of conjugal transfer of RK2, with one *Eco*RI site, and a derivative, with four *Eco*RI sites, with results consistent with a reduction of about 15-fold per site, as well.

Nonetheless, it is evident that in many instances conjugal transfer is little affected by restriction, despite a formidable block against some other mode of transfer (5, 20, 36, 41, 55, 66). Plasmid-encoded antirestriction defenses (16, 32) or the induction by conjugation of a restriction-free state (28) can explain only some of these cases. The rest might be explained if the single-stranded state of DNA transferred by conjugation affords special protection to incoming DNA, but this is a difficult notion to accept. Even when single-stranded DNA is introduced directly, by transformation, it is equally as sensitive to restriction by *Eco*B as is double-stranded DNA (6), despite the fact that *Eco*B, like many other endonucleases (49, 56), has little or no measurable activity towards single-stranded DNA in vitro (38).

Conjugal transfer of DNA may gradually overcome restriction through prolonged periods of conjugation (10, 55), owing

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either to the accumulation of rare successes from sustained bombardment, as in multiple phage infection (50), or to the eventual saturation of host defenses (73). Quantitative differences in the effect of restriction on conjugal transfer efficiency observed from one experiment to the next (e.g., the results of *Ava*I restriction in Fig. 4 and 5) may be explained by conditions that inadvertently favored or reduced the viability of donor *E. coli* on the medium used in matings. Investigators attempting to introduce DNA into restriction-competent recipients by conjugation might well bear in mind the desirability of extended mating periods.

The low activity of *Asp*(7120)III, described here, and the requirement for repeated freeze-thaw cycles to obtain that activity would explain why the enzyme was overlooked by previous workers (19). Judging by the ability of pRL623 to protect plasmids against restriction by *Anabaena* sp. strain PCC 7120, in vivo as well as in vitro, it is unlikely that there are other restriction activities to be found. The strain therefore appears to possess exactly the same enzyme complement as *Anabaena* sp. strain PCC 7118 (46, 59), adding further evidence to the hypothesis that the two widely studied strains are two isolates of the same organism (57).

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