

# Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen-fixing filamentous cyanobacteria

(*Anabaena*/plasmid RP-4/plasmid pBR322/restriction sites/photosynthesis)

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**ABSTRACT** Wild-type cyanobacteria of the genus *Anabaena* are capable of oxygenic photosynthesis, differentiation of cells called heterocysts at semiregular intervals along the cyanobacterial filaments, and aerobic nitrogen fixation by the heterocysts. To foster analysis of the physiological processes characteristic of these cyanobacteria, we have constructed a family of shuttle vectors capable of replication and selection in *Escherichia coli* and, in unaltered form, in several strains of *Anabaena*. Highly efficient conjugative transfer of these vectors from *E. coli* to *Anabaena* is dependent upon the presence of broad host-range plasmid RP-4 and of helper plasmids. The shuttle vectors contain portions of plasmid pBR322 required for replication and mobilization, with sites for *Anabaena* restriction enzymes deleted; cyanobacterial replicon pDU1, which lacks such sites; and determinants for resistance to chloramphenicol, streptomycin, neomycin, and erythromycin.

Many filamentous cyanobacteria fix dinitrogen aerobically within specialized cells called heterocysts that differentiate at semiregular intervals along the filaments. All cyanobacteria are capable of oxygenic photosynthesis. Genetic methods usable for study of nitrogen fixation, differentiation, pattern formation, and photosynthesis in these organisms have long been sought.

Several unicellular cyanobacteria can be transformed by DNA in the growth medium (1-3). Shuttle vectors, plasmids able to replicate in *Escherichia coli* and in an alternative host, have been constructed for two such strains, *Anacystis nidulans* strain R2 and *Agmenellum quadruplicatum* PR-6 (4-6). An *Anacystis* gene cloned in a shuttle vector in *E. coli* was returned to the cyanobacterium by transformation (7). To date, no reproducible transformation system is known for filamentous cyanobacteria.

Conjugation provides an alternative approach to transfer of cloned DNA. RP-4 and related plasmids can transfer themselves or derivatives of themselves into a wide range of Gram-negative bacteria (8-10). Cyanobacteria have the structure and wall composition of Gram-negative bacteria (11). Delaney and Reichelt (12) have described a very low frequency transfer of R68.45, closely related to RP-4, into a unicellular cyanobacterium, but establishment of RP-4 in a filamentous cyanobacterium has not been observed.

One can utilize the conjugal properties of RP-4 without demanding that that plasmid replicate in a new host. Several conjugative plasmids can promote the transfer of derivatives of pBR322 between strains of *E. coli* (13) or from *E. coli* to other Gram-negative bacteria (14, 15) so long as the *bom* (basis of mobility) region of the transferred plasmid is left intact and requisite *trans*-acting factors are present. Such factors are provided by pDS4101 (ColK::TnI) or pGJ28 (ColD Km<sup>r</sup>; ref. 13). Shuttle vectors based on pBR322 may thereby assume the wide conjugal range of RP-4.

We report the construction of a hybrid between pBR322 and plasmid pDU1 (16) from the filamentous cyanobacterium *Nostoc*. Because restriction endonucleases present in strains of cyanobacteria (17) apparently reduce retention of DNA transferred into those cyanobacteria (4, 18), the hybrid plasmid was restructured to eliminate sites for restriction enzymes present in several strains of *Anabaena*. Additional antibiotic-resistance determinants were added, lest an organism be unable to use any one such determinant. The derivatives of the hybrid plasmid proved to be shuttle vectors, capable of RP-4-mediated transfer into several strains of *Anabaena* and of replication in those strains.

## MATERIALS AND METHODS

*Anabaena* sp. PCC 7120, *Anabaena* sp. U. Leningrad strain 458 (PCC 7118), *Anabaena* sp. U. Tokyo M-131, and *Nostoc* sp. PCC 7524 were grown with nitrate as described (19). *Anacystis nidulans* strain R2 was grown in medium BG-11 (20). *E. coli* was grown in L broth, supplemented as appropriate with none, one, or two of the following: 25 µg of chloramphenicol (Cm) per ml, 10-25 µg of streptomycin (Sm) per ml, 50 µg of kanamycin (Km) per ml, 50 µg of ampicillin (Ap) per ml. Plasmids were isolated from cyanobacteria by established methods (5, 21, 22) or minor variations thereof, and from *E. coli* strains HB101 and Gm48 (*dam*<sup>-</sup> *dcm*<sup>-</sup>) essentially as described (23). Plasmids were digested with restriction enzymes from New England BioLabs and Bethesda Research Laboratories in buffers recommended by the suppliers, or slight variations thereof. Recombinant DNA techniques were standard (23).

For mating experiments, *E. coli* HB101 containing a cyanobacterial hybrid plasmid and either pGJ28 or pDS4101, and *E. coli* J53(RP-4) were grown in shaken test tubes, overnight, at 37°C, then separately diluted 0.25 ml:10 ml of antibiotic-supplemented L broth. After approximately 2.5 hr of growth in shaken flasks, 0.75-ml portions were harvested (12,000 × g, 1 min). The bacteria were washed with L broth, mixed or not with other strains, centrifuged as before, and resuspended in 60 µl of L broth, permitting unselected transfer of RP-4 to HB101 to take place. *Anabaena* strains becoming light-limited at 30°C, but still growing actively, were concentrated 20-fold (12,000 × g, 1 min); *Anacystis* was concentrated to 10<sup>9</sup> cells per ml. Nuclepore filters sterilized in water (24) were set atop solidified cyanobacterial media (19, 20) supplemented with 5% (vol/vol) L broth, in Petri plates. Twenty-microliter portions of each of a series of 1:10 dilutions of the cyanobacterial suspensions were then streaked onto the filters, and the streaks were dried. Two-microliter portions of bacterial suspensions were spotted atop the dried

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Tc, tetracycline; <sup>r</sup>, resistant (resistance); <sup>s</sup>, sensitive (sensitivity); bp, base pair(s); kb, kilobase pair(s).

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streaks of cyanobacteria, and the Petri plates were incubated at *ca.* 23°C and low light intensity for 4–6 hr. The filters were then transferred to unsupplemented, solidified cyanobacterial medium and were set at 30°C and high light intensity (*ca.* 3500 erg cm<sup>-2</sup> s<sup>-1</sup>). When inducible resistance to erythromycin (Em) was to be transferred, the medium contained a sub-inhibitory concentration (0.1 µg/ml) of Em. Twenty-four hours after the filters were spotted with bacteria [this period of time provided for expression of antibiotic resistance (24)], the filters were transferred to solidified cyanobacterial medium supplemented with selective concentrations of antibiotics and returned to 30°C and high light intensity.

## RESULTS

**Cloning Plasmid pDU1 from *Nostoc* PCC 7524.** Plasmid pDU1, linearized by partial digestion with *EcoRV*, was inserted into the *EcoRV* site of plasmid pBR322 and used to transform *E. coli* HB101. One clone contained a 10.6-kilobase-pair (kb) plasmid, denoted pVW1, which was chosen for detailed study. The three *EcoRV* fragments, 4.1, 1.2, and 0.98 kb, of pDU1 are retained in pVW1 (Fig. 1A) without rearrangement (Fig. 1B); pBR322 has been inserted within the largest *HindIII* fragment (Fig. 1B, lanes 1 and 4).

Plasmid pVW1 and derivatives of it (see below) were tested for restriction by additional endonucleases. Cloned pDU1 contained restriction sites shown in Fig. 2, plus at least 4 sites for *Aha* III (from a cyanobacterium) and at least 10 sites for *Hha* I, *Apa* I, *Ava* I, *Ava* II, *Avr* II, *Bal* I, *Bam*HI, *Ban* II, *Bgl* II, *Bst*EII, *Cla* I, *Eco*RI, *Hgi*AI, *Kpn* I, *Nae* I, *Nar* I, *Nco* I, *Nde* I, *Nru* I, *Pst* I, *Sac* II, *Sal* I, *Sau*96I, *Sph* I, *Sst* I, and *Stu* I failed to cut the pDU1 portion of pVW1. Enzymes that failed to cut pVW1 did cut λ DNA when λ DNA was added to the pVW1 restriction reaction mixtures. Cloned pDU1 was found to contain sites for *Bcl* I, *Pvu* I (*Xor* II), *Pvu* II, and *Hae* III (because the plasmid is cut by *Xma* III) although these enzymes did not cut pDU1 from *Nostoc* (16).

**Construction of Conjugal Shuttle Vectors.** Plasmid pVW1 has two disadvantages for use as a cloning vector. First, it has eight *Ava* II sites and one *Ava* I site at which it could be restricted upon transfer to numerous strains of *Anabaena* (17). Second, its selectable markers, resistance to tetracycline (Tc) and Ap, are not well suited for conjugal transfer to cyanobacteria: Tc breaks down rapidly in the light used for growth of cyanobacteria; and resistance to Ap is mediated by a β-lactamase, secretion of which by a donor strain could protect a recipient strain.

Resistance to Cm encoded by pBR328 (25) has neither of these disadvantages. A *Sau*3AI fragment containing the entire structural gene for Cm acetyltransferase was therefore ligated into the *Bam*HI site of pVW1 (Figs. 3 and 4). Plasmids in three Cm<sup>r</sup> Ap<sup>r</sup> Tc<sup>s</sup> transformants of *E. coli* all contained the same insert in the same orientation (transcription antiparallel to that of the Ap<sup>r</sup> gene). One of the plasmids was designated pVW1C.

To remove the *Ava* I and *Ava* II sites from pVW1C, this plasmid was digested with *Ava* II, treated with alkaline phosphatase, extracted with phenol, digested with *Sau*96I, ligated, and used to transform *E. coli*; cells were selected for resistance to Cm. The 5' extensions of the *Sau*96I sites are trinucleotides and therefore not self-complementary. Theoretically, possible repolymerizations (see refs. 25–27) must contain *Sau*96I fragments [in pBR322 coordinates (26, 27)] from bp 173 to bp 524, containing the *EcoRV* site, hence the cloned pDU1, and the erstwhile *Bam*HI site, hence the Cm<sup>r</sup> determinant; from bp 1951 to bp 3411, containing *bom* and the vegetative origin; and either bp 4346 through bp 0 to bp 172 (containing a unique site for *Cla* I) or bp 3412 to bp 3490, either providing a necessary linker. Plasmid pRL1, used for subsequent constructions, has the structure shown in Fig. 4. It lacks sites for *Ava* I, *Ava* II, and *Avr* II. Determinants for

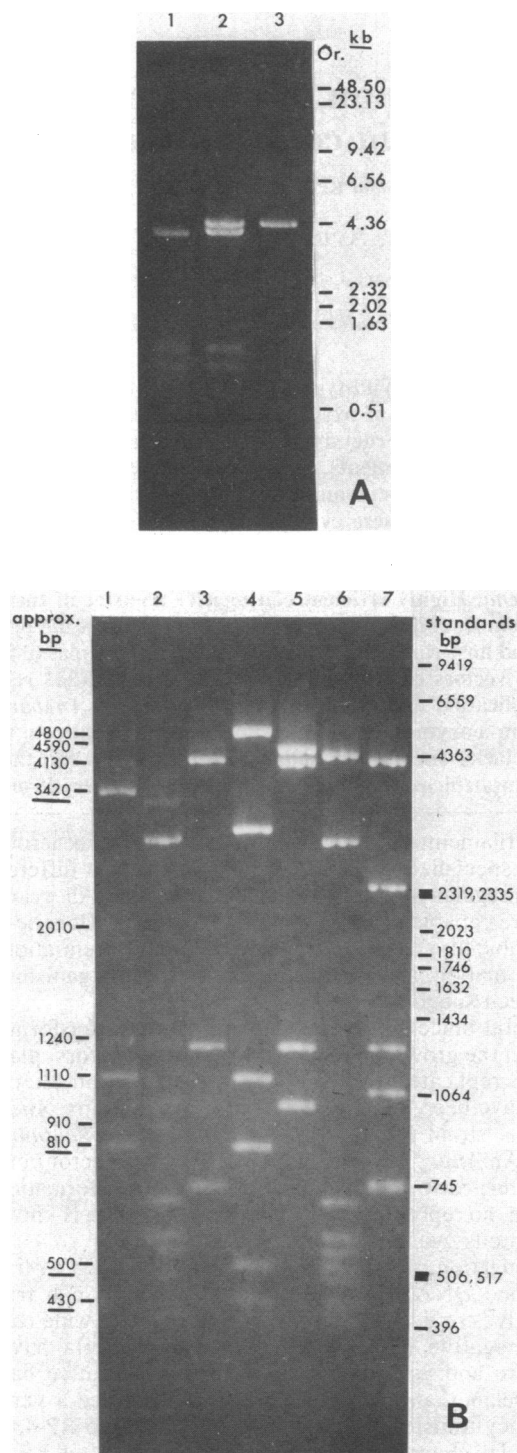


FIG. 1. Electrophoretograms (agarose 0.6% in A, 1.2% in B) of products of digestion of pDU1 (A, lane 1; B, lanes 1–3), pVW1 (A, lane 2, B, lanes 4–7), and pBR322 (A, lane 3). The positions of molecular weight markers, derived by digestion of pBR322 with *Hinf*I, *Bgl* I, and *Ava* II and of λ DNA with *Hind*III, are shown. (A) Restriction by *EcoRV*. (B) Restriction by *Hind*III [lanes 1 (partial digest) and 4], *EcoRV* (lane 5), *Hind*III and *EcoRV* [lanes 2 (partial digest) and 6], and *Bgl* I and *EcoRV* (lanes 3 and 7). Bands at *ca.* 3420, 1110, 810, 500, and 430 base pairs (bp) (underlines) in lane 1 of B are seen in limit digests. Bands at *ca.* 4590 ( $\approx$  3420 + 1110), 4130 ( $\approx$  3420 + 500), 2010 ( $\approx$  810 + 1110), 1240 ( $\approx$  810 + 430), and 910 ( $\approx$  500 + 430) bp confirm the sequence (ref. 16; Fig. 2) of *Hind*III fragments in pDU1. Equality of certain fragment lengths for pDU1 and pVW1 (lanes 1 and 4, 2 and 6, and 3 and 7) is possible only if the sequence and relative orientations of the *EcoRV* fragments of pDU1 are retained in pVW1.

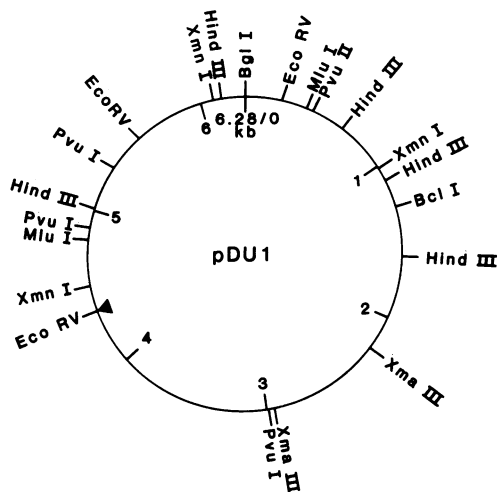


FIG. 2. Restriction map of pDU1. ▲, Site of insertion of pBR322 in pVW1. The orientation of this map matches that in ref. 16 but is the reverse of the orientation in Fig. 4.

Sm<sup>r</sup> (28) in pRL5, Km<sup>r</sup>/neomycin (Nm)<sup>r</sup> (29, 30) in pRL6, and Em<sup>r</sup> (31) in pRL8 (Figs. 3 and 4) introduce sites for *Ava* I (Sm<sup>r</sup>), *Ava* II (Sm<sup>r</sup>, Km<sup>r</sup>, Em<sup>r</sup>), and *Ava* III (Em<sup>r</sup>) but not for *Avr* II.

**Conjugal Transfer of Vectors to Cyanobacteria.** Three elements suffice for the conjugal transfer of pBR322 and derived plasmids: a suitable conjugative plasmid—e.g., RP-4; a plasmid such as pGJ28 or pDS4101 to provide necessary transfer functions; and the pBR322 derivative (13). The latter

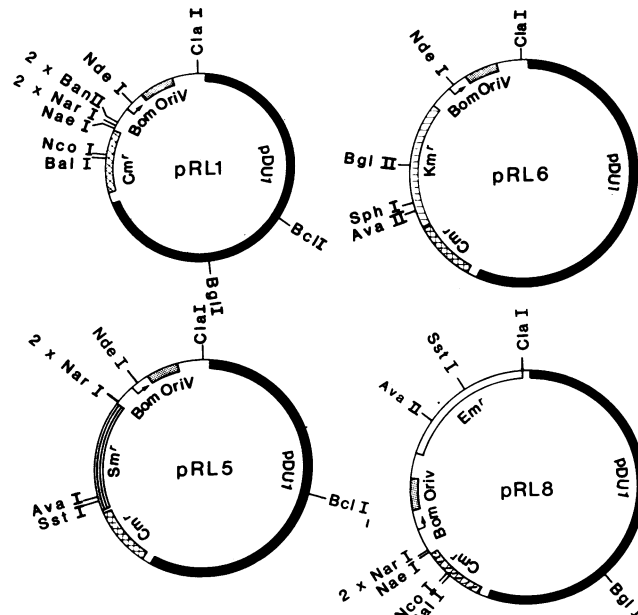


FIG. 4. Maps of plasmids pRL1, pRL5, pRL6, and pRL8, showing potential cloning sites; the portions derived from pDU1 (solid bars) and pBR322 (lines), the Cm<sup>r</sup> fragment from pBR322 (cross-hatched bars), the Sm<sup>r</sup> fragment from R300B (lined bars), the Km<sup>r</sup> fragment from Tn5 (hatched bars), and the Em<sup>r</sup> fragment from pE194 (empty bars), and the positions of the origin of vegetative replication, *oriV* (stippled bars) and the *bom* region (arrows).

two elements were combined prior to mating by using pRL1, pRL5, and pRL8 to transform HB101(pGJ28) and using pRL6 to transform HB101(pDS4101). Triparental matings were then performed (8) with strains of cyanobacteria, *E. coli* J53(RP-4), and *E. coli* HB101 containing pRL1 or derivative and the helper plasmid. As illustrated in Fig. 5, only matings in which all elements were present resulted in substantial growth of cyanobacteria different from unmated controls. At high concentrations of cyanobacterial inoculum, the cyanobacteria often grew in the presence of antibiotic (Fig. 5D). Occasional colonies that developed in the presence of only one or two plasmids were not studied further. Upon attempted conjugal transfer of pRL1V, pRL5V, or pRL6V (i.e., pRL1, -5, or -6 from which the pDU1 portion had been excised) to strains of *Anabaena*, and of pRL1, -5, or -6 to *Anacystis*, growth was observed only so long as viable donor bacteria were present (see *Discussion*). The pRL1 progenitor, pVW1C, containing one *Ava* I and eight *Ava* II sites, was—unlike pRL1—not transferred efficiently (Fig. 5D).

To characterize their content of plasmids, the presumptively exconjugant cyanobacteria were freed of the auxotrophic strains of *E. coli* used, by streaking on metabolite-free cyanobacterial medium. Portions of single colonies were transferred to L agar. Colonies appearing axenic were grown in liquid medium with antibiotic. Plasmids were isolated from dense suspensions, the purity of which was confirmed by transfer to L broth and L agar. The presumptive exconjugants showed plasmid profiles that included bands corresponding to the hybrid plasmids (Fig. 6). Transformation of *E. coli* HB101 by plasmid-containing extracts yielded a large number of transformants. The transformed plasmids were isolated, restricted, and subjected to electrophoresis (Fig. 7). We found that pRL1, -5, and -6 could be transferred from *E. coli* to *Anabaena*, and back, without alteration; pRL8 may be less stable. Evidence of plasmid transfer and replication was obtained with combinations of cyanobacteria, plasmids, and selective agents shown in Table 1. Resistances to Sm,

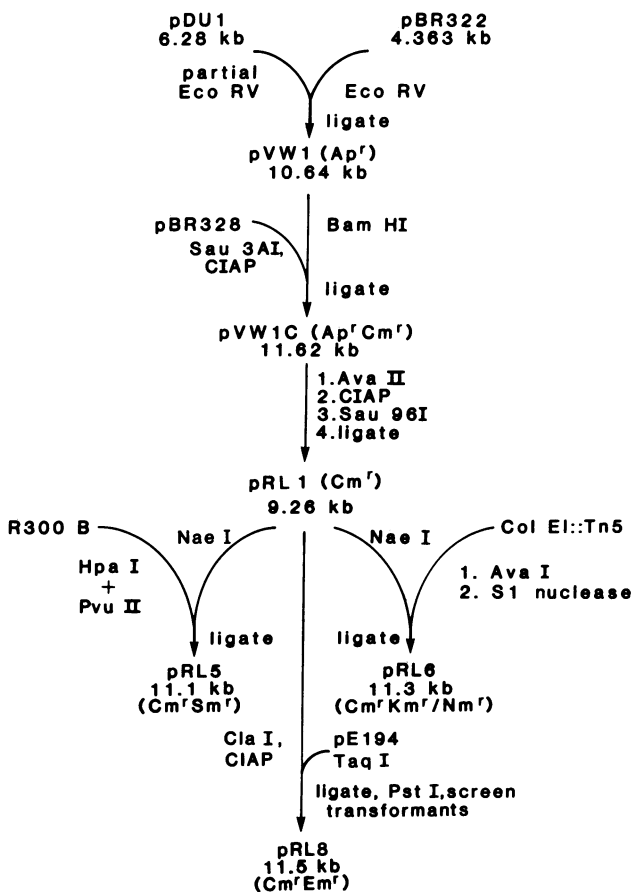


FIG. 3. Construction of pRL1, pRL5, pRL6, and pRL8. CIAP, calf intestinal alkaline phosphatase.

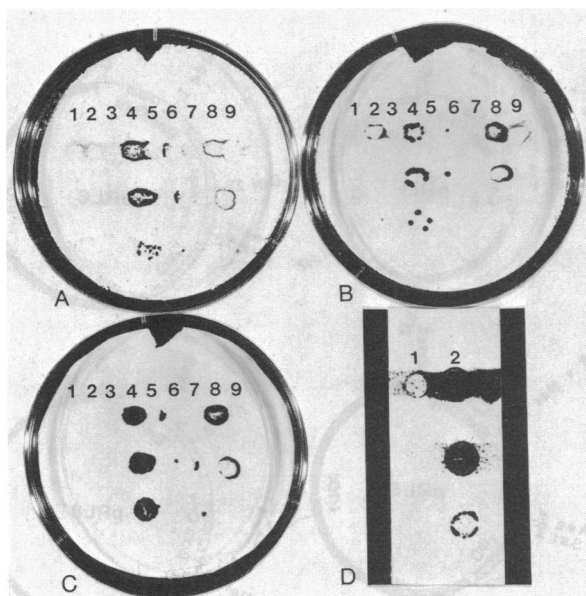


FIG. 5. Typical initial results of mating experiments. Nucleopore filters were streaked (from top to bottom) with successive 1:10 dilutions of suspensions of *Anabaena* strains 7120 (A), 458 (B), and M-131 (C and D). The streaks in A, B, and C were dotted with 2- $\mu$ l portions of (from left to right) suspensions of *E. coli* strains containing plasmids RP-4 (spots 1); pRL6 (spots 2); pDS4101 (spots 3); RP-4, pRL6, and pDS4101 (spots 4); pRL6 and pDS4101 (spots 5); RP-4 and pDS4101 (spots 6); RP-4 and pRL6 (spots 7); RP-4, pDS4101, and pRL6V (which is pRL6 lacking pDU1) (spots 8); and only L broth (spots 9). The streaks in D were dotted with 2- $\mu$ l portions of suspensions of *E. coli* strains containing RP-4, pGJ28, and either pVW1C (spots 1) or pRL1 (spots 2). The media contained Nm at 25 (A and C) or 10 (B)  $\mu$ g/ml, or Cm at 20  $\mu$ g/ml (D). D is magnified  $\times 1.24$  relative to A, B, and C.

Nm, and Em characteristic of pRL5, -6, and -8 were not conferred by pRL1.

In experiments such as Fig. 5 A-C, the ratio of exconjugant cyanobacterial colonies to the number of cyanobacterial cells subtended by a spot of bacteria was about  $10^{-3}$ . However, when *Anabaena* M-131 was fragmented by cavitation to an average length of 1.3 cells per filament before conjugal transfer of pRL1 (for which no restriction is expected), the ratio of exconjugant colonies (developing in the presence of Cm at 20  $\mu$ g/ml) to total colonies (developing in the absence of Cm) was approximately 0.03.

### DISCUSSION

We have demonstrated RP-4- and helper plasmid-dependent transfer of pBR322-based plasmids across the wide taxonomic gap from *E. coli* to *Anabaena*. It was fortunate that our hybrid vectors, with a *Nostoc* replicon, were able to replicate in strains of *Anabaena*. They apparently cannot repli-

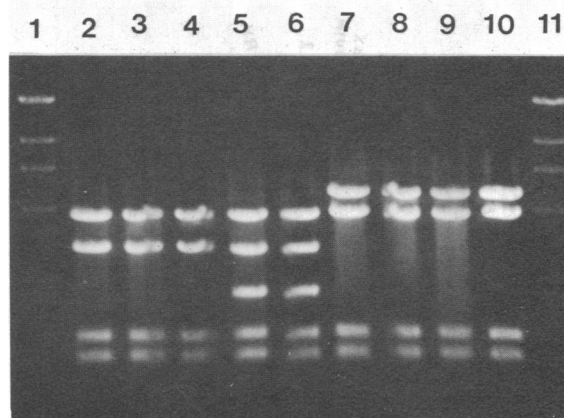


FIG. 7. Electrophoretograms of *EcoRV* digests of DNA extracted from *E. coli* after transformation with extracts of axenic *Anabaena* cultures that had been mated with *E. coli* strains bearing pRL1 (lanes 2 and 3), pRL5 (lane 5), and pRL6 (lanes 7-9). Lanes 4, 5, and 10, authentic pRL1, -5, and -6; lanes 1 and 11, *HindIII* digest of  $\lambda$  DNA. *Anabaena* strains: lanes 2 and 7, 7120; lanes 3, 5, and 8, M-131; and lane 9, 458.

cate in *Anacystis*. When matings are performed from *E. coli* to *Anacystis* with pRL1, -5, or -6, or when matings are performed to *Anabaena* in which pRL1, -5, or -6 is replaced by pRL1V, pRL5V, or pRL6V—i.e., without inclusion of pDU1—growth of the cyanobacteria is often seen. However, once freed of *E. coli*, the cyanobacteria no longer form colonies in the presence of antibiotic. A mobilizable derivative of shuttle vector pSG111 (32), which contains an *Anacystis* replicon, could be transferred to *Anacystis* by conjugation and could replicate there (unpublished data). It appears that plasmids transferred to the cyanobacteria by conjugation can confer antibiotic resistance upon them, but that only when the resistance-determinant is on a replicon functional in the particular cyanobacterium is resistance maintained in the absence of further infusion of plasmids.

Our success was apparently dependent in part on the reduction or elimination of restriction as an impediment to retention of conjugally transferred DNA (see Fig. 5D). This was possible because the specificities of restriction enzymes of numerous strains of cyanobacteria have been identified, and M-131, in particular, appears to have only isoschizomers of *Ava* I and *Ava* II; because the Cm<sup>r</sup> gene from pBR328 and essential parts of pBR322 lack sites for *Ava* I and *Ava* II; and because the cyanobacterial replicon pDU1 present in our hybrid vector lacks sites for *Ava* I and *Ava* II.

It may not be a matter of chance that pDU1 lacks such sites. *Nostoc* sp. PCC 7524, from which pDU1 is derived, has five restriction endonucleases, *Nsp*(7524)I through V (33). *Nsp*(7524)III (which cuts C<sup>+</sup>Y-C-G-R-G; Y = pyrimidine; R = purine) is an isoschizomer of *Ava* I. The specific-

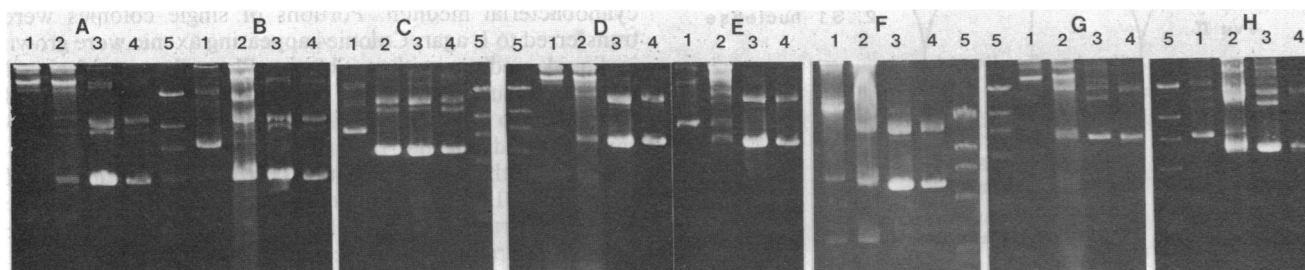


FIG. 6. Electrophoretograms of DNA extracted from *Anabaena* (strain 7120 in A, D, and G; strain M-131 in B, C, E, and H; strain 458 in F) both unmated (lanes 1) and mated (lanes 2) with *E. coli* bearing pRL1 (A and B), pRL5 (C), pRL6 (D, E, and F), or pRL8 (G and H) and then rendered axenic. Lanes 3, DNA extracted from *E. coli* after transformation with a portion of the extract in lanes 2; lanes 4, authentic pRL1 (A and B), pRL5 (C), pRL6 (D, E, and F), or pRL8 (G and H); and lanes 5, *HindIII* digest of  $\lambda$  DNA.

Table 1. Selective conditions for plasmid transfer to cyanobacteria

Anabaena strain	Plasmid and antibiotic, $\mu\text{g/ml}$			
	pRL1 Cm	pRL5 Sm	pRL6 Nm	pRL8 Em
M-131	20, 30	3, 5, 10	10, 25	2, 5
458			10	
7120	5, 10		25	5

ity of *Nsp*(7524)IV (G<sup>+</sup>G-N-C-C; ref. 33), an isoschizomer of *Sau*96I, includes all *Ava* II sites (G<sup>+</sup>G-A<sup>+</sup>-C-C). Unlike *Hpa* II (C<sup>+</sup>C-G-G; ref. 16), *Hae* III (G-G<sup>+</sup>C-C; see above), and *Hha* I (G-C-G<sup>+</sup>C; see above), *Sau*96I fails to cut pDU1 ( $P \approx 10^{-11}$ ). Furthermore, *Ban* II, *Hgi*AI, and *Sph* I, the specificities of which are subsumed by those of *Nsp*(7524)I and -II, all fail to cut unmodified pDU1 ( $P < 0.004$ ). Finally, cloned chromosomal DNA from *Anabaena* ATCC 29413 (unpublished data) and *Anabaena* 7120 (34–38) is statistically deficient in sites for *Ava* I, and *Ava* I and II, respectively, for which they have isoschizomers (17). It must be considered that an organism may evolve to minimize the number of target sites in its DNA for its own restriction endonucleases.

Demonstration of high-frequency genetic transfer to *Anabaena* opens the way to analysis of cyanobacterial nitrogen fixation and development by the techniques of modern biochemical genetics. In addition, it provides a major tool for study of oxygenic photosynthesis.

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