JOURNAL OF BACTERIOLOGY, June 1985, p. 1339–1341
0021-9193/85/061339-03$02.00/0

Vol. 162, No. 3

Identification of Facultatively Heterotrophic, N_2-Fixing Cyanobacteria Able To Receive Plasmid Vectors from *Escherichia coli* by Conjugation

ENRIQUE FLORES AND C. PETER WOLK*

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

Received 10 October 1984/Accepted 20 March 1985

Plasmid vectors transferable by conjugation from *Escherichia coli* to obligately photoautotrophic strains of *Anabaena* spp. are also transferred to and maintained in heterotrophic, filamentous cyanobacteria of the genus *Nostoc*. These organisms can be used for the genetic analysis of oxygenic photosynthesis, chromatic adaptation, nitrogen fixation, and heterocyst development.

Cyanobacteria are unique in that they are procaryotes that are able to perform oxygenic photosynthesis (14, 15). Use of these organisms should therefore permit application of the powerful techniques of bacterial genetics to the analysis of water-splitting photosynthesis. Such analysis would be greatly facilitated by the use of facultatively heterotrophic strains, e.g., for transposon mutagenesis. With few known exceptions (1, 15), the facultatively heterotrophic cyanobacteria are filamentous strains, many of which are capable also of heterocyst differentiation (14, 15), photosynthetic fixation of dinitrogen within the heterocysts (11), and chromatic adaptation of the photosynthetic apparatus (4, 15).

Shuttle plasmid vectors have recently been developed that can be transferred efficiently by conjugation from *Escherichia coli* to several obligately photoautotrophic members of the genus *Anabaena* of filamentous cyanobacteria (16). As one approach to a genetic analysis of photosynthesis, we have sought to identify facultatively heterotrophic strains amenable to conjugative genetic transfer. Our efforts have focused mainly on strains of the genus *Nostoc* because many of them are facultatively heterotrophic as well as capable of aerobic nitrogen fixation (12, 15) and because our shuttle vectors incorporate replicon pDU1 from a strain (PCC 7524) of *Nostoc*.

The organisms tested were *Nostoc* spp. strains ATCC 27895 (PCC 6720), ATCC 27896 (PCC 6310), ATCC 27897 (PCC 6302), ATCC 29107 (PCC 7416), ATCC 29133 (PCC 73102), and ATCC 29150 (PCC 7107) (see reference 12) and *Fischerella muscicola* UTEX 1829 (see reference 10). All of these strains are capable of aerobic fixation of dinitrogen, and all have been reported to be facultatively photoheterotrophic (i.e., capable of growth in the light with sugar as the source of reductant when the water-splitting photosystem II is inactivated by dichlorophenyl dimethyl urea) or heterotrophic. They were routinely grown at 30°C in the light in medium AA/8 (6). For growth in darkness, liquid media AA/8 (6) and BG-11 (12) or 1% agar-solidified media AA (6) and BG-11 (12) were tested in each case with and without nitrate and supplemented with sugars (see below) at 25 to 50 mM. Growth rates were calculated from the increase in the concentration of chlorophyll (8) of axenic cultures. Filaments of strain ATCC 29150 were fragmented by cavitation (17) and washed. The fragments, counted with a hemacytometer, were grown in the dark in top, soft (0.5%) agar supplemented with fructose and with 2 mM NH_4Cl (buffered with 4 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid–NaOH [pH 7.5]), N_2, or nitrate as the nitrogen source.

Shuttle vectors were used (16) that bear determinants for resistance to chloramphenicol (Cm) alone (plasmid pRL1) or to Cm and streptomycin (Sm; pRL5), neomycin or kanamycin (Nm or Km; pRL6), or erythromycin (Em; pRL8). These plasmids were transferred from *E. coli* HB101 by use of conjugative plasmid RP-4 and helper plasmids pGJ28 (for pRL1, pRL5, and pRL8) and pDS4101 (for pRL6), essentially as described previously (16). After 2 weeks, presumptive exconjugants were freed from contaminating *E. coli* by restreaking on selective medium. Axenic colonies (see reference 16) were transferred to antibiotic-supplemented liquid medium, and plasmids were isolated from these cultures as described by Simon (13), after rechecking that contaminant *E. coli* cells were absent by plating samples of the culture on L agar. Other techniques for manipulation of plasmid DNA were standard (9).

As an initial screen for possible transfer of shuttle vectors from *E. coli* to cyanobacteria, we compared the growth of cyanobacteria, in spots on a filter that had been exposed to strains of *E. coli* (suspended in L broth) containing the plasmids, with the growth in spots that had simply received L broth. With the following strains and shuttle vectors and at the indicated concentrations of antibiotic, the cyanobacteria grew in the spot containing *E. coli* but not in the corresponding control spot without the *E. coli* mixture: *Nostoc* sp. strain ATCC 27896 and pRL5 (1 to 5 μg of Sm per ml), pRL6 (10 to 25 μg of Nm per ml), or pRL8 (5 μg of Em per ml); *Nostoc* sp. strain ATCC 29107 and pRL8 (5 μg of Em per ml); *Nostoc* sp. strain ATCC 29133 and pRL5 (1 μg of Sm per ml) or pRL6 (25 μg of Nm per ml); *Nostoc* sp. strain ATCC 29150 and pRL6 (10 to 25 μg of Nm per ml) or pRL8 (2 to 5 μg of Em per ml); and (in some but not all experiments) *F. muscicola* 1829 and pRL6 (25 μg of Nm per ml) or pRL8 (1 to 2 μg of Em per ml). The highest frequencies of recovery of presumptive exconjugants were obtained with *Nostoc* sp. strain ATCC 27896. In a particular mating experiment in which filaments fragmented by cavitation (17) to an average length of 1.2 cells were used, the ratio of presumptive exconjugants (obtained in the presence of

* Corresponding author.

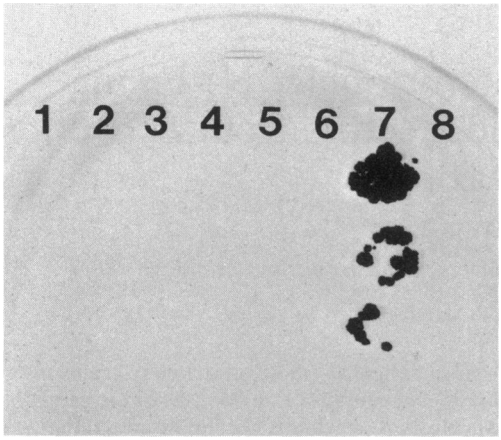

FIG. 1. Mating of *Nostoc* sp. strain ATCC 29133 with strains of *E. coli* containing different combinations of plasmids. Streaks of 20 μl of suspensions of *Nostoc* sp. (100 [top], 10 [middle], and 1 [bottom] μg of chlorophyll per ml; 3×10^6 to 4×10^6 cells per μg of chlorophyll) were dotted with *E. coli* strains containing the following plasmids: (1) RP-4; (2) pDS4101; (3) pRL6; (4) RP-4 and pDS4101; (5) RP-4 and pRL6; (6) pDS4101 and pRL6; and (7) RP-4, pDS4101 and pRL6; or (8) with L broth and no *E. coli*. The medium contained 25 μg of Nm per ml. The photograph was taken 1.5 months after inoculation.

antibiotic) to CFU (in controls without antibiotic) was 1×10^{-3} to 3×10^{-3} for transfers involving pRL6 and pRL8.

To test whether the growth of cyanobacteria in the presence of *E. coli* actually resulted from the conjugative transfer of the shuttle vectors to the cyanobacteria, mating experiments were carried out involving all combinations of one, two, or all of the three plasmids used for a given transfer. A representative result of such an experiment is shown in Fig. 1 for the particular case of transfer of pRL6 to *Nostoc* sp. strain ATCC 29133: growth of the cyanobacterium in the selective medium was observed in the spot of *E. coli* containing the three plasmids required for conjugation (RP-4, pDS4101, and pRL6) but not in those spots containing any subset of those plasmids. Equivalent results were obtained in tests involving *Nostoc* sp. strain ATCC 29150 and pRL6 (25 μg of Nm per ml), *Nostoc* sp. strain ATCC 27896 and pRL6 (25 μg of Nm per ml) or pRL5 (5 μg of Sm per ml), and *Nostoc* sp. strain ATCC 29133 and pRL5 (1 μg of Sm per ml). Such tests were not performed with pRL8.

Because the cyanobacteria were more sensitive to antibiotics in liquid than on solid medium, liquid media were supplemented with lower (but still selective) concentrations of antibiotic than were solid media. Among the presumptive exconjugants, *Nostoc* sp. strains ATCC 27896, ATCC 29133, and ATCC 29150 that had been mated with *E. coli* containing pRL6 grew especially well in axenic, antibiotic-containing liquid cultures (containing Nm at 2.5, 8.3, and 2.5 μg/ml, respectively). Exconjugant *Nostoc* sp. strain ATCC 29150 bearing pRL6 could be grown in the dark in a medium supplemented with 50 mM fructose and 2.5 μg of Nm per ml.

An analysis of plasmid preparations from liquid cultures of mated cyanobacteria is presented in Fig. 2. In each instance, the presumptive exconjugant contains a plasmid absent from the respective wild-type strain, and the results of digestion with *Eco*RV establish that the extra plasmid DNA corresponds to the shuttle vector pRL6. Furthermore, *E. coli* ED8654 could be transformed to Km^r with these plasmid preparations from the cyanobacteria and pRL6 recovered from the transformants (Fig. 2).

Plasmid preparations were also made from axenic, liquid cultures of presumptively exconjugant cyanobacteria from other matings, but the results obtained were not as clear as

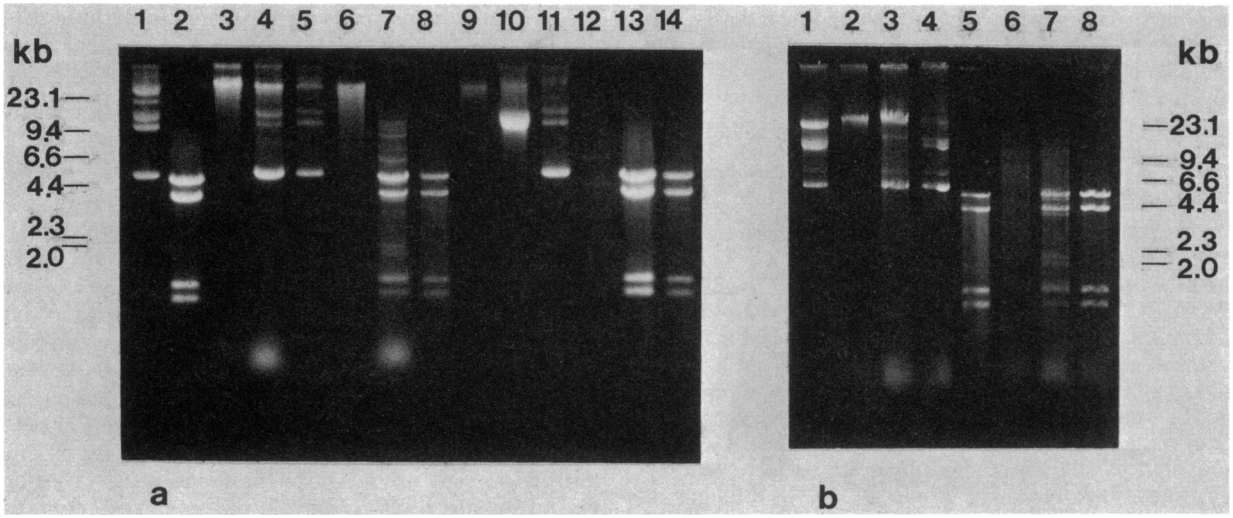

FIG. 2. Electrophoretograms of DNA extracted from wild-type and exconjugant strains of *Nostoc* spp. (a) Lanes contained DNA from the following: wild-type *Nostoc* sp. strain ATCC 27896 (lanes 3 and 6), axenic *Nostoc* sp. strain ATCC 27896 derived from a mating with *E. coli* bearing pRL6 (lanes 4 and 7), *E. coli* ED8654 transformed with the latter preparation (lanes 5 and 8), wild-type *Nostoc* sp. strain ATCC 29133 (lanes 9 and 12), axenic *Nostoc* sp. strain ATCC 29133 derived from a mating with *E. coli* bearing pRL6 (lanes 10 and 13), and *E. coli* ED8654 transformed with the latter preparation (lanes 11 and 14). Lanes 1 and 2 show authentic pRL6. These preparations were unrestricted (lanes 1, 3 through 5, and 9 through 11) or were restricted with *Eco*RV (lanes 2, 6 through 8, and 12 through 14). (b) Lanes contained the following: authentic pRL6 (lanes 1 and 5); DNA from dark-grown, wild-type *Nostoc* sp. strain ATCC 29150 (lanes 2 and 6); DNA from dark-grown, axenic *Nostoc* sp. strain ATCC 29150 derived from a mating with *E. coli* bearing pRL6 (lanes 3 and 7); DNA from *E. coli* ED8654 transformed with the latter preparation (lanes 4 and 8). These preparations were unrestricted (lanes 1 through 4) or restricted with *Eco*RV (lanes 5 through 8). The positions of molecular weight markers are shown. Kb, kilobase.

those just described. A new band of plasmid DNA comigrating with authentic pRL8 during electrophoresis in agarose gels was observed in preparations from *Nostoc* sp. strain ATCC 29107 (grown with 0.5 μg of Em per ml) and *F. muscicola* 1829 (grown with 0.2 μg of Em per ml) that had been mated with *E. coli* containing pRL8 (data not shown). However, these preparations gave rise to no Cm^r transformants of *E. coli*. We have not sought to establish whether the Em^r determinant from pRL8 is present in the new plasmids of the presumptive exconjugants.

The results presented in this paper show that shuttle vector pRL6, encoding resistance to Cm and Nm (or Km), is transferred by conjugation to and is autonomously maintained in *Nostoc* sp. strains ATCC 27896, ATCC 29133, and ATCC 29150. All three of these strains grow as discrete colonies on solid medium in the light with N_2 as the nitrogen source. They are therefore suitable for genetic study of heterocyst formation and aerobic nitrogen fixation. Moreover, all three are facultative heterotrophs with the following generation times in shaken liquid cultures in the dark: *Nostoc* sp. strain ATCC 27896, 2 to 3 days with glucose or (after a prolonged lag) fructose and 4 days with sucrose; *Nostoc* sp. strain ATCC 29133, 5 to 6 days with glucose or fructose and 4 days with sucrose; and *Nostoc* sp. strain ATCC 29150, 1.5 days with fructose and 4 days with sucrose (we did not observe growth with glucose). We note, however, that growth as single colonies on solid medium in the dark is not always observed with sugars which permit growth of the same organism in the dark in liquid. Although *Nostoc* sp. strain ATCC 27896 has been reported to grow with glucose on solid medium in the dark (7), we consistently observed the formation of single colonies in the dark only with *Nostoc* sp. strain ATCC 29150. Even after fragmentation (17) to an average of 4.5 cells per filament, this strain can grow both in the light and in the dark as discrete colonies in top (0.5%) agar with a plating efficiency (CFU per fragment) of close to 100%. (It has not been established whether transformable, facultatively heterotrophic unicellular cyanobacteria [1, 2, 5] can be grown from single cells in the dark.) *Nostoc* sp. strain ATCC 29150 could therefore be suitable for genetic studies of photosynthesis. Finally, *Nostoc* sp. strains ATCC 29133 (4) and ATCC 27896 (3) both show chromatic adaptation and may therefore be useful for the genetic analysis of this phenomenon.

This work was supported by the U.S. Department of Energy under contract DE-AC02-76ER01338. E.F. was supported during the first part of this work by Fundación J. March (Spain) and thereafter by a Fulbright/Ministerio de Educación y Ciencia (Spain) fellowship.

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