A mobilizable shuttle vector for the cyanobacterium *Plectonema boryanum*

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Plasmid pSUP5011 contains a pMB1 origin of replication, an origin of transfer (*oriT*), and genes encoding resistance to the antibiotics ampicillin, chloramphenicol and kanamycin. pSUP5011 was conjugally mobilized from *Escherichia coli* into the non-heterocystous, filamentous, nitrogen-fixing cyanobacterium *Plectonema boryanum* UTEX 594 in the presence of a helper plasmid, RP4. Transconjugant cyanobacteria selected for resistance to kanamycin, ampicillin and chloramphenicol showed a variety of DNA rearrangements in pSUP5011. One such plasmid continued to show characteristic rearrangements following subsequent transfers into the cyanobacterium. A stable plasmid useful as a shuttle vector was isolated.

Introduction

Cyanobacteria are a unique group of eubacteria in which photosynthesis is accompanied by oxygen evolution, as in the case of higher plants. Certain cyanobacteria can also fix molecular nitrogen. Many nitrogen-fixing cyanobacteria compartmentalize nitrogen fixation in heterocysts to protect the nitrogenase complex from photosynthetically evolved oxygen. However, several cyanobacteria, including those in the genera *Plectonema* (Weare & Benemann, 1974), *Synechococcus* (Mitsui *et al.*, 1986; Grobbelaar *et al.*, 1987), *Gloeotheca* (Gallon *et al.*, 1988) and *Oscillatoria* (Gallon *et al.*, 1991) do not differentiate heterocysts, but achieve temporal separation of photosynthesis and nitrogen fixation. *Plectonema boryanum* UTEX 594 shows a cyclic rise and fall of photosynthesis alternating with a cyclic appearance of nitrogen fixation when it is grown microaerobically under nitrogen starvation conditions in continuous light (Misra & Tuli, 1992; Rai *et al.*, 1992). The genetic basis of the cyclic regulation of photosynthetic oxygen evolution is not known.

Genetic studies on non-heterocystous nitrogen-fixing cyanobacteria are limited by the absence of methods for gene transfer. Cloning vectors available for some of the nitrogen-fixing heterocystous cyanobacteria (Wolk *et al.*, 1984; Murray & Wolk, 1991) have a narrow host range (Flores & Wolk, 1985) and do not function in *P.*

boryanum (R. K. Iyer & R. Tuli, unpublished). These vectors are based on cyanobacterial replicons. Mobilization and stable maintenance of plasmids with a broad-host-range replicon of the IncQ group was recently reported in certain cyanobacteria (Kreps *et al.*, 1990; Sodo *et al.*, 1992). This report describes isolation of a recombinant plasmid that can be efficiently mobilized from *Escherichia coli* to *P. boryanum* UTEX 594. Preliminary results have been briefly reported (Tuli *et al.*, 1990).

Methods

Organisms and growth conditions. *P. boryanum* strain UTEX 594 is from the University of Texas collection. In other collections it is identified as PCC 73110, ATCC 29407 and M-9.2.1 (Houmard & Tandeau de Marsac, 1988). It was grown in BG11 medium (Rippka *et al.*, 1979) at 25 °C, bubbled with air under continuous illumination from white fluorescent light at 2360 lux (measured with a lux meter, model ANA 999, Tokyo Photoelectric Co.). *P. boryanum* UTEX 594 does not grow in BG11 liquid medium in the presence of 0.5 µg ampicillin ml⁻¹, 2 µg kanamycin ml⁻¹ or 20 µg chloramphenicol ml⁻¹ when inoculated at an OD₇₀₀ of 0.15. A culture adjusted to OD₇₀₀ 0.15 and spotted on BG11 medium solidified with 1% (w/v) agar also failed to grow at these antibiotic levels. However, occasional growth was noticed when the cyanobacterium was inoculated at 10- to 20-fold higher cell density and challenged with these antibiotic concentrations. Hence concentrations of the antibiotics were maintained at the above levels, and cultures were inoculated at OD₇₀₀ 0.15.

E. coli S17-1 (Simon *et al.*, 1983a) is a donor strain derived from *E. coli* 294 (*recA supE44 hsdR endA1 pro thi-1*) (Simon *et al.*, 1983b). It carries a chromosomally integrated RP4-2, which is a derivative of RP4 with a deletion in *TnI*. The kanamycin-resistance gene in RP4-2 is inactivated by a *Tn7* insertion and the tetracycline-resistance gene is non-functional due to a *Mu* insertion. *E. coli* S17-1 RP4-2 (Tc::Mu) (Km::Tn7) was grown in Luria Broth (LB) in the presence of 100 µg streptomycin ml⁻¹. The plasmid pSUP5011 (see Fig. 2) is a derivative

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of pBR325. An IncP-specific site for mobilization (*oriT*) was cloned into the unique *Bam*HI site of Tn5; the resulting Tn5-Mob was inserted into the gene for tetracycline resistance in pBR325 (Simon *et al.*, 1983a). After transfer into *E. coli*, pSUP5011 confers resistance to ampicillin, chloramphenicol and kanamycin. Both *E. coli* S17-1 and *E. coli* 294(pSUP5011) were kind gifts from Dr Ursula B. Priefer (University of Bielefeld, Germany). *E. coli* S17-1(pSUP5011) was prepared by transformation and used in biparental matings with *P. boryanum* UTEX 594. *E. coli* AB1157 (Sassanfar & Roberts, 1991) transformed with pSUP5011 was used as a Rec⁺ control to demonstrate the inability of pSUP5011 alone (in the absence of RP4) to allow the growth of *P. boryanum* UTEX 594 in the presence of antibiotics on conjugation spots.

Mating between *E. coli* and *P. boryanum*. A conjugation procedure similar to that described by Wolk *et al.* (1984) was adopted. The donor *E. coli* S17-1(pSUP5011) was grown overnight in the presence of ($\mu\text{g ml}^{-1}$) streptomycin (100), kanamycin (50), ampicillin (50) and chloramphenicol (15). The next day, it was diluted 40-fold into fresh LB containing the antibiotics and grown with shaking for about 3 h at 37 °C to an OD₆₀₀ of 0.45. To prepare the donor for mating, 0.75 ml of this culture was washed and resuspended in 60 μl of antibiotic-free LB. The recipient *P. boryanum* UTEX 594 was grown in BG11 medium for 7 d to an OD₇₀₀ of 0.7. It was prepared for mating by concentrating the culture 20-fold in BG11 medium.

A mating mixture was prepared by mixing 5 μl of the donor with 5 μl of the recipient. The mixture was spotted onto a nitrocellulose (Millipore HAWP, 0.45 μm) disc placed on solidified BG11 medium, supplemented with 5% (v/v) LB. The nitrocellulose discs were washed overnight in distilled water and autoclaved in advance. Tenfold serial dilutions (10^{-1} , 10^{-2} and 10^{-3}) of the cyanobacterium were included in the mating mixtures (Fig. 1) because highly concentrated suspensions showed some growth even in the presence of presumed inhibitory levels of the antibiotics. After 4 h, the nitrocellulose disc was lifted and placed on a BG11 plate devoid of LB and incubated in low light (1290 lux) for about 24 h. The disc was then lifted and placed on a fresh BG11 plate containing selective concentrations of kanamycin ($10 \mu\text{g ml}^{-1}$), ampicillin ($10 \mu\text{g ml}^{-1}$) and chloramphenicol ($50 \mu\text{g ml}^{-1}$). The conjugation plate was incubated in low light (1290 lux) for 3 d and then transferred to high light (2360 lux) to allow rapid growth of the transconjugant *P. boryanum* colonies in the mating spots. The cyanobacterial growth from a conjugation spot was picked after 7–10 d and inoculated into 5 ml BG11 liquid medium supplemented with $10 \mu\text{g ml}^{-1}$ kanamycin. After a week, the cyanobacterial growth was subcultured into BG11 containing kanamycin, ampicillin and chloramphenicol at concentration of 10, 10 and $50 \mu\text{g ml}^{-1}$, respectively.

Mapping of sites for restriction enzymes was done by labelling the ends with ³²P before and after cutting with restriction enzymes (singly and in combination). The labelled fragments were resolved on 0.8–2% (w/v) agarose gels, as appropriate. Southern hybridization of genomic DNA prepared from the cyanobacterium was done under conditions of high stringency (42 °C in the presence of 50%, v/v, formamide) on Gene Screen membranes as detailed by the manufacturer (New England Nuclear). Low-stringency hybridizations in certain experiments were carried out under similar conditions except that the temperature during hybridization and washings was reduced to 30 °C to allow approximately 35–38% mismatch.

Total DNA from *P. boryanum* transconjugants was prepared by a method modified from Felkner & Barnum (1988). The cyanobacterial culture grown to mid-exponential phase in BG11 medium was washed in 10 mM-Tris, 1 mM-EDTA, pH 8.0. One gram of cell pellet was suspended in 4 ml sucrose buffer (50 mM-Tris, pH 8.0, 100 mM-EDTA, 25%, w/v, sucrose) and incubated for 1 h at 37 °C after adding lysozyme to a final concentration of 5 mg ml^{-1} . It was then treated with proteinase K (final concentration $100 \mu\text{g ml}^{-1}$) in the presence of 2.5%

(w/v) SDS at 37 °C until the suspension became clear. The cleared lysate was extracted with phenol and chloroform prior to precipitation of DNA as described by Felkner & Barnum (1988). The cyanobacterial DNA was used in Southern hybridizations or for transformation of *E. coli* according to general methods in Sambrook *et al.* (1989).

Results

The result of conjugation experiments showed mobilization of pSUP5011 from *E. coli* to *P. boryanum* UTEX 594 and expression of the three antibiotic resistance genes resident on pSUP5011. Spots of *P. boryanum* UTEX 594 mixed with *E. coli* S17-1 (Fig. 1, row 1), and with *E. coli* AB1157(pSUP5011) (Fig. 1, row 2) showed no growth in the antibiotic-containing medium. However, the spots containing conjugation mixture of *P. boryanum* UTEX 594 with *E. coli* S17-1(pSUP5011) (Fig. 1, row 4) began to show cyanobacterial growth within 5–10 d. Since no transconjugant cyanobacterium appeared in the mating spots containing *E. coli* AB1157(pSUP5011), the *trans*-acting mobilization factors and the conjugational functions of RP4 integrated in the chromosome of *E. coli* S17-1 are necessary for the transfer of pSUP5011 into *P. boryanum* UTEX 594.

Fig. 1. Result of conjugation between *E. coli* and *P. boryanum* UTEX 594. The cyanobacterial strain mixed with *E. coli* S17-1 (row 1), *E. coli* AB1157(pSUP5011) (row 2) or *E. coli* AB1157(pKAC51) (row 3) was unable to grow on nitrocellulose filters placed on BG11 medium containing ($\mu\text{g ml}^{-1}$) kanamycin (10), ampicillin (10) and chloramphenicol (50). However, *P. boryanum* UTEX 594 mixed with *E. coli* S17-1(pSUP5011) (row 4) and *E. coli* S17-1(pKAC51) (row 5) showed photoautotrophic growth implying conjugal transfer of the plasmids, the latter being more efficient. Vertical columns 2–4 contain 10-fold serial dilutions of the *P. boryanum* UTEX 594 culture used in column 1.

Fig. 2. Restriction maps of the plasmids pSUP5011, pKAC58, pKAC581 and pKAC51. E, Nd, H, Hp, Sm, Nr, S, B, RV and C refer to sites for the restriction enzymes *EcoRI*, *NdeI*, *HindIII*, *HpaI*, *SmaI*, *NruI*, *SalI*, *BamHI*, *EcoRV* and *ClaI*, respectively.

Fig. 3. Autoradiograph showing hybridization of a pSUP5011 DNA probe to total DNA from transconjugant *P. boryanum* UTEX 594. (a) *P. boryanum* UTEX 594(pKAC51) uncut (lane 1) and *HindIII*-cut DNA (lane 2). (b) *P. boryanum* UTEX 594(pKAC58) uncut (lane 1), *HindIII*-cut (lane 2) and *SmaI*-cut (lane 3) DNA. The positions of the molecular size standards, λ DNA cut with *HindIII*, are shown on the left and right for (a) and (b) respectively.

The presumptively transconjugant cyanobacterial patches were picked from the 10^{-3} conjugation spots and inoculated into 5 ml BG11 liquid medium containing $10 \mu\text{g}$ kanamycin ml^{-1} . The cyanobacterial growth appearing in about 7 d was diluted 50-fold in fresh BG11 containing ampicillin, kanamycin and chloramphenicol at 10, 10 and $50 \mu\text{g}$ ml^{-1} respectively. After three to four subcultures, the cyanobacterium was unequivocally free

of any bacteria, which was confirmed by placing 25 μl spots of the culture on LB-agar medium and on BG11 medium supplemented with casein amino acids (0.05%, w/v) and glucose (0.5%, w/v). Thus, the patches of *P. boryanum* UTEX 594 appearing on the conjugation plates comprised stable transconjugants.

After several months of maintenance in BG11 medium supplemented with the three antibiotics, total DNA was prepared from the axenic transconjugant cyanobacterium grown in 500 ml of the selective BG11 medium. The DNA preparation was used to transform *E. coli* HB101 and the transformants were selected on LB agar containing (μg ml^{-1}) any one of the three antibiotics – kanamycin (50), ampicillin (50) and chloramphenicol (15). Nearly 30% of the *E. coli* transformants were resistant to all three antibiotics, while about 70% were resistant to kanamycin and ampicillin but not to chloramphenicol. Restriction analyses of the plasmids recovered from some of the transformed *E. coli* showed three types of plasmids (Fig. 2), none of which was identical to pSUP5011. The 12.5 kb pKAC51 and the 13.75 kb pKAC58 were mobilizable in *E. coli* by RP4 and conferred resistance to all three antibiotics. However, the 4.7 kb pKAC581 was not mobilizable by RP4 and conferred resistance to kanamycin and ampicillin but not to chloramphenicol. Both pKAC51 and pKAC58 could also be mobilized from *E. coli* S17-1 into *P. boryanum* UTEX 594.

Remobilization of pKAC51 into *P. boryanum* UTEX 594 resulted in its establishment without further rearrangements. This was substantiated both by Southern hybridization and by the phenotype conferred by the plasmids recovered after transformation of *E. coli* with DNA prepared from *P. boryanum* UTEX 594(pKAC51)

Fig. 4. (a) Autoradiograph showing stability of pKAC51 in *P. boryanum* UTEX 594(pKAC51). The strain was subcultured in antibiotic-free BG11 for three months and then grown in BG11 containing the three antibiotics before DNA extraction. Uncut (lane 1), *Bam*HI-cut (lane 2), *Hind*III-cut (lane 3), *Eco*RV-cut (lane 4) and *Sma*I-cut DNA (lane 5) were probed with pKAC51. (b) Autoradiograph showing absence of homology between pKAC51 DNA probe and *P. boryanum* UTEX 594 wild-type DNA. *P. boryanum* UTEX 594 uncut (lane 2), *Eco*RV-cut (lane 3) and *Hind*III-cut (lane 4) DNA. Weak hybridization was seen with the 23 kb band of *Hind*III-cut λ DNA used as a size marker (lane 1). The positions of the molecular size standards, λ DNA cut with *Hind*III, are shown on the left and right for (a) and (b) respectively.

transconjugants. Southern hybridizations using pSUP5011 as probe showed that pKAC51 was present in the transconjugant cyanobacterium as a stable, circular, extrachromosomal molecule. Plasmid pSUP5011, used as a probe with total DNA of *P. boryanum* UTEX 594(pKAC51) cut with restriction enzymes, showed homology only to fragments diagnostic of pKAC51 (Fig. 3a). However, following remobilization of pKAC58 into *P. boryanum* UTEX 594, a smaller plasmid, pKAC581, appeared in addition to pKAC58. Plasmids pKAC581 and pKAC58 could be demonstrated in transconjugants both by Southern hybridization (Fig. 3b) and by transformation of *E. coli*. Uncut total DNA prepared from transconjugant *P. boryanum* UTEX 594 carrying pKAC51 or pKAC58 showed faint plasmid bands (masked by the background of genomic DNA) in addition to the native plasmids in ethidium-bromide-stained gels (data not shown), suggesting that these plasmids are maintained in the cyanobacterium at a low copy number.

pKAC51 was mobilized more efficiently into the cyanobacterium than pSUP5011. Efficiency of conjugal transfer was difficult to estimate since *P. boryanum*

UTEX 594 does not plate efficiently on serial dilutions and shows a spreading type of growth. However, the patches appearing on conjugation plates clearly showed a more confluent growth at the spots corresponding to mating with pKAC51 (Fig. 1, row 5) as compared to those of mating with pSUP5011 (Fig. 1, row 4). Transconjugant *P. boryanum* UTEX 594(pKAC51) did not lose the plasmid rapidly in the absence of selection pressure. After several subcultures, over a period of three months, in BG11 devoid of antibiotics, the transconjugant cyanobacterium grew well after a lag of about 48 h following a challenge with all three antibiotics. On Southern hybridization using pKAC51 as a probe, such cultures showed the presence of pKAC51 without any further rearrangements (Fig. 4a). Even at low stringency of hybridization and washings (both at 30 °C), pKAC51 showed no homology with the chromosomal DNA or native plasmids (Vachhani *et al.*, 1992) of wild-type *P. boryanum* UTEX 594 (Fig. 4b). Under such conditions pKAC51 showed a low level of hybridization with the 23 kb *Hind*III fragment of phage λ in the lane corresponding to the DNA size standards. The results indicate that pKAC51 establishes itself in *P. boryanum* UTEX 594 as an autonomous molecule without interacting with the cyanobacterial genome.

Discussion

We have shown that *in vivo* rearrangements following mobilization of pSUP5011 into the non-heterocystous nitrogen-fixing cyanobacterium *P. boryanum* UTEX 594 led to the formation of plasmids that replicated in the cyanobacterium. One such plasmid, pKAC51, was phenotypically similar to pSUP5011, but had a different restriction map. Since pKAC51 is mobilizable by RP4, expresses three different antibiotic-resistance determinants, and replicates in both *E. coli* and *P. boryanum* UTEX 594, it could serve as a useful shuttle vector for genetic studies of the regulation of carbon and nitrogen metabolism.

Successful establishment of pKAC51 in *P. boryanum* UTEX 594 is apparently dependent upon several *in vivo* rearrangements that occur in the progenitor plasmid, pSUP5011. Instability of the insertion elements in pSUP5011 and problems related to the expression of alien genes and replication could be among the reasons that necessitate the rearrangements leading to the establishment of this plasmid in the cyanobacterium. Mechanistically, the rearrangements appear quite complex, but they seem to follow a pattern. For instance, although the restriction maps of pKAC58 and pKAC581 were quite dissimilar, the latter appeared from the former in repeated independent matings. The regions of pKAC51, pKAC58 and pKAC581 that function as

replicon in *P. boryanum* UTEX 594 are not known. The parental plasmid pSUP5011 contains a narrow-host-range replicon *oriV* from pMB1 for replication in *E. coli*. This replicon does not function in *Anabaena* 7120 (Wolk *et al.*, 1984). In the unicellular cyanobacterium *Anacystis nidulans* PCC 6301, pBR322 has been reported to establish itself following transformation (Daniell *et al.*, 1986). However, such an ability has been attributed to homologous recombination with chromosomal DNA (Daniell *et al.*, 1989). Following transformation with pBR322, homology-based integration of *bla* into the chromosome of *Agmenellum quadruplicatum* PRG has also been reported (Buzby *et al.*, 1985). Within the limits of detection by Southern hybridization at low stringency, genomic DNA from *P. boryanum* UTEX 594 showed no homology with either pSUP5011 or pKAC51. Hybridization and washing under conditions that allowed 35–38% mismatch detected no homology of pKAC51 with total DNA or native plasmids in the transconjugant *P. boryanum* UTEX 594(pKAC51). The results imply that a heterologous replicon in pKAC51 functions in *P. boryanum* UTEX 594. Among broad-host-range plasmids, an IncQ plasmid has been reported to replicate in *Synechocystis* sp. PCC 6803 (Kreps *et al.*, 1990) and a few other cyanobacteria (Sodo *et al.*, 1992). It is not clear if pSUP5011 contains DNA sequences from the chromosome of *E. coli* S17-1 that facilitate its establishment in the cyanobacterium or if all the rearrangements occur after transfer into *P. boryanum* UTEX 594, leading to the formation of a functional replicon under selective pressure.

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