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Conjugal gene transfer in filamentous cyanobacteria

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Though cyanobacterial gene manipulation is very difficult and time consuming than bacteria due to constraints like longer generation time, cryptic genetic material, lack of colony formation (filamentous cyanobacteria). But, the time has come when genetic improvement has become inevitable for the better utilization of biotechnologically important cyanobacteria like *Anabaena, Nostoc, Oscillatoria, Calothrix, Westiellopsis, Spirulina.* Cyanobacteria are prokaryotes with gram-negative bacteria like cell wall and eukaryotes like aerobic photosynthetic apparatus. Cyanobacteria have a high market value as natural therapeutic and colouring substance besides, its conventional use as protein suppliment and nitrogen fixers. To bring down the product cost, extensive cyanobacterial genetic manipulations are badly needed. Cyanobacterial microbiologists were handicapped due to lack of reproducible gene-transfer system in filamentous cyanobacteria till Wolk *et al.* for the first time reported successful gene transfer *in Anabaena* using shuttle vector pVW_1 and its derivatives, broad host range conjugal plasmid RP_4 of incompatibility group (IncP) and helper plasmid pGS101/pGJ28. The use of these plasmids made the conjugation possible in cyanobacteria. Indeed conjugation is the only technique now available for gene transfer in filamentous cyanobacteria, viz. *Anabaena, Nostoc, Fremyella, Fischerella.* It has opened the way for gene manipulation studies in other biotechnologically important filamentous cyanobacteria like *Spirulina* and *Westiellopsis*, provided one takes for surety of conjugal contact, degradation of transferred DNA from host's (recipients) restriction enzyme digestion, and the transferred gene (plasmid) is capable of replication or integration into the recipient cell.

CYANOBACTERIA are a unique group of prokaryotes with both oxygenic photosynthesis and the capacity to fix elemental nitrogen. An understanding of the coexistence of these two primary biosynthetic processes thus promises a closer application in higher plants than other nitrogen-fixing microbes, which either have anoxygenic photosynthesis or are heterotrophic. Besides, cyanobacteria have a high market value as a source for natural therapeutic and colouring substances[1–3]. To bring down the product cost, extensive cyanobacterial gene manipulations are needed.

Investigations on gene organization and function in these microbes (cyanobacteria) are limited because of their slow growth, in many cases multicellular nature of their colony forming units, the spreading tendency of many of them on agar plates and nonavailability of simple and efficient gene transfer systems. The identification and characterization of a 'classic' mechanism for genetic recombination in cyanobacteria lags behind tremendously in comparison to other prokaryotes. For

a long time cyanobacteria have been thought to be devoid of any genetic transfer system until workers[4-6] reported their success in achieving gene transfer in *Synechococcus*, *Cylindrospermum*, and *Anabaena* respectively, though later their findings were challenged[7]. Bazin[8] demonstrated occurrence of gene transfer by isolating double resistant mutants after cocultivation of colony raised mutants of *Synechococcus* resistant to streptomycin or polymyxin B. Again it could not be established whether the gene transfer had occurred by transformation or conjugation. Later on, a large number of papers were published claiming success in gene transfer through transformation or conjugation in the filamentous cyanobacterium *Nostoc* for nitrogen fixation, heterocyst formation, resistance to antibiotics, antimetabolites, herbicides and pesticides[9-12]. Further work for obtaining stable recombinant clones using purified DNA has confirmed the gene transfer by transformation[13,14]. Success was also obtained in transferring herbicide resistance of *Synechocystis* to filamentous *Nostoc* either by mixing the cultures or by isolated DNA[15]. Gene transfer studies in cyanobacteria through transformation were confined to *Anacystis nidulans* R_2 and *Agmenellum quadruplicatum*[13,16-19], while reports of transformation in filamentous cyanobacteria including *Nostoc* await confirmation. At present no reproducible transformation system has been developed for the filamentous forms, except in *Anabaena* strain M131 using electroporation[20]. Transformation has also been reported for *Nostoc*, but the technique adopted is not being discussed[21].

For filamentous cyanobacteria, conjugation appears to be an alternative approach for genetic manipulation, for which first success was reported in *Anabaena* using shuttle vector pVW1 and its derivatives, broad host range conjugal plasmid RP4 of incompatibility group (IncP) and helper plasmid pGS4101/pGJ28 (ref. 22). This success on conjugal gene transfer and its stable maintenance was later observed in strains of *Nostoc* sp. strain ATCC 27896 (PCC 6310) and pRL5 (1–5 μg Sm per ml), pRL6 (10–25 μg Nm per ml) or pRL8 (5 μg Em per ml); *Nostoc* sp. strain ATCC 29107 (PCC 7416) and pRL8 (5 μg Em per ml); *Nostoc* sp. strain ATCC 29133 (PCC7 3102) and pRL5 (1 μg Sm per ml) or pRL6 (25 μg Nm per ml); *Nostoc* sp. strain ATCC 29150 (PCC 7107) and pRL6 (10–25 μg Nm per ml) or pRL8 (2–5 μg Em per ml); and in some *Fischerella musciola* UTX 1829 and pRL6 (25 μg Nm per ml) or pRL8 (1–2 μg Em per ml). These *Nostoc* strains were able to fix nitrogen and to grow heterotrophically as well as facultative heterotrophically, i.e. they were capable of growth in presence of light with sugar as a source of reductant when water splitting photosystem II is inactivated by dichlorophenyldimethylurea. *Nostoc* sp. strain ATCC 27896, 29133 and 29150 appeared as the best filamentous cyanobacteria for genetic analysis of aerobic nitrogen fixation and heterocyst formation[23]. *Nostoc* sp. strain ATCC 29150 was capable of chromatic adaptations, i.e. it can grow in both light and dark conditions as discrete colonies in top 0.5% agar with 100% plating efficiency which is not yet reported in facultative heterotrophic unicellular cyanobacteria and thus *Nostoc* sp. strain ATCC 29150 appears to be a highly suitable organism for genetic studies on photosynthesis. Plasmids containing Tn5 and its derivatives have also been successfully transferred from *E. coli* to *Anabaena* 7120 and *Plectonema boryanum* through conjugation[24-26]. Plasmids have been transferred by conjugation in unicellular forms like *Anacystis* (*Synechococcus*) and *Aphanocapsa* (Synechocystis)[27,28].

As the cyanobacterial shuttle vector carrying the genes for bacterial luciferase *lux A* and *lux B* with its own promoter showed almost nil activity, the promoter for structural genes for ribulose biphosphate carboxylase or nitrogenase from *Anabaena* has been inserted upstream from luciferase genes and transferred through conjugation in the recipient cell. Production of light in exconjugants has confirmed expression of bacterial luciferase very well in *Anabaena*[26,29]. Since then, conjugation technique is used by many cyanobacteriologists for understanding basic cyanobacterial molecular biology[30-40]. This also attracted the attention of cyanobacterial microbiologists to work on nitrogen fixation, oxygenic photosynthesis as well as synthesis of pigments, antibiotics, fatty acids and toxins in biotechnologically important cyanobacteria like *Anabaena*, *Nostoc*, *Oscillatoria*, *Calothrix*, *Westiellopsis*, *Plectonema* and *Spirulina* but appreciable success has not yet been achieved.

The other most popular and feasible mode of gene transfer, i.e. transduction, appeared to be unsuccessful inspite of the reports of specific cyanobacteriophages[41-43]. Not only this, conjugation is preferred to transformation for introducing large DNA segments because of nonoccurrence of random cutting of introduced DNA unlike transformation, ie. conjugation increases the efficiency of transferring nonhomologous segments. It also increases the probability of getting single recombinant products (merodiploids) rather than double recombination products (gene replacement). It also avoids the problem of extracellular nucleases. Conjugation has permitted the complimentation of mutants with cosmid libraries providing an opportunity to identify mutated genes, which may be specific to only cyanobacteria for which heterologous probes are not available, e.g. genes related to development of heterocyst[32,44].

Molecular basis of conjugation

Conjugation is a biological phenomenon in lower organisms for direct transfer of DNA from a donor into

a recipient cell via physical contact, which is mediated by two groups of plasmid coded genes, transfer genes (*tra*) and mobilizing genes (*mob*). The transfer gene cluster is composed of more than 12 genes and is responsible for synthesis of pili (which help in physical contact) and other surface components. The *mob* functions are defined by at least two regions on plasmid DNA. The first region results in nick formation for conjugal transfer. It is called *nic/bom* region or ori^T. The second region provides DNA nicking protein that specifically recognizes *bom* site (ori^T) and is known as *mob* region. tra^+ mob^+ plasmids (Figure 1,*b*) are functionally transferable and mobile, e.g. sex factor F and other plasmids belonging to incompatibility class Inc, F-I, F-II, F-III, F-IV. tra^- mob^+ plasmids (Figure 1,*a*) lacking the transfer function are nonconjugative but these can be mobilized if another conjugative plasmid is present in the same cell, e.g. Col E_1 and Cloacin DF_{13} ($CloDF_{13}$) which can be mobilized by F factor and F factor like plasmids. tra^- mob^- plasmids are neither conjugative nor can they be mobilized, except in conditions when part of *mob* region coding mobilizing protein is present along with another plasmid coding for functional *mob* protein which acts in *trans* on mob^- plasmid. But the *nic/bom* site is always a *cis* acting element and cannot be provided by another plasmid[45].

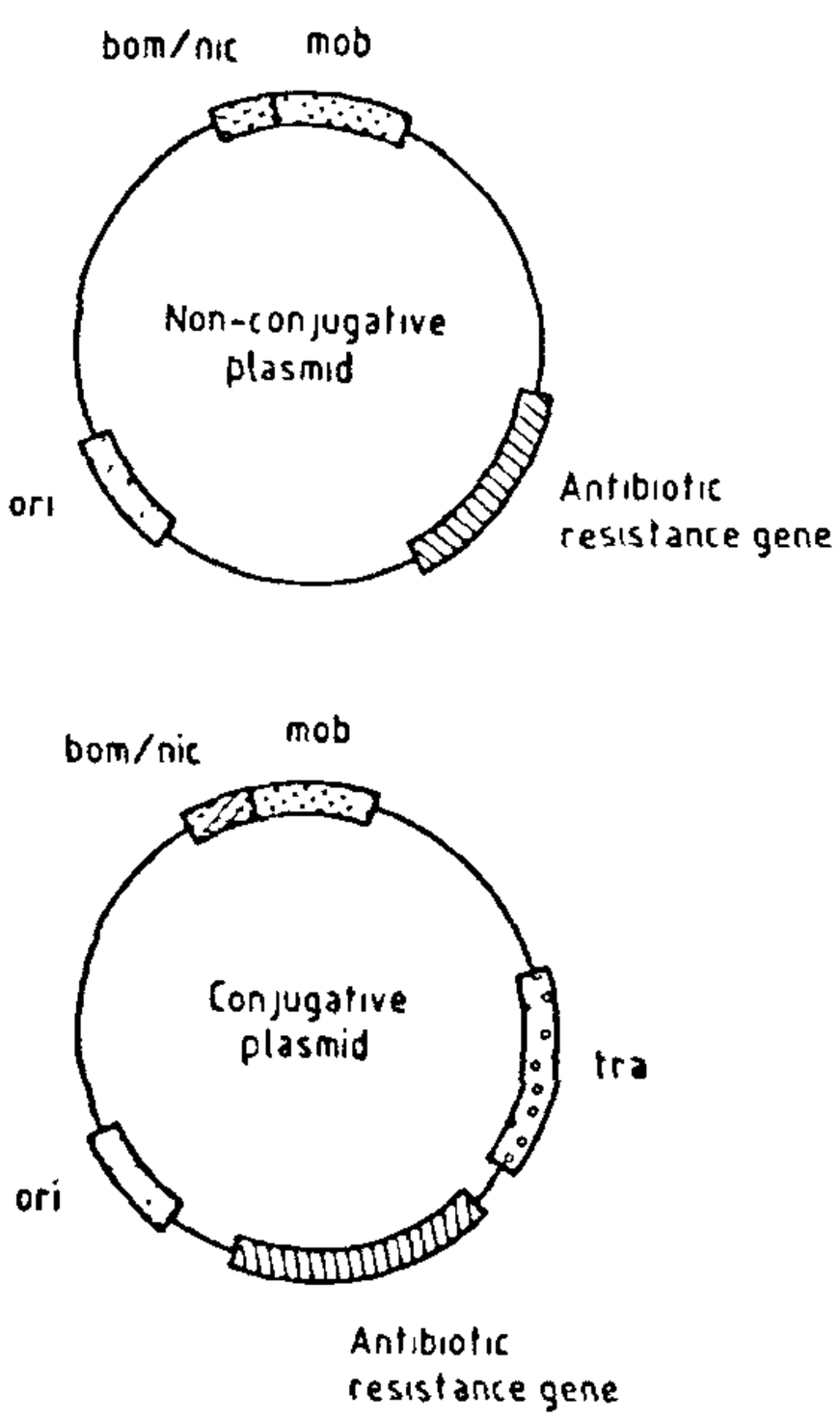

Figure 1. Diagrammatic representation of non conjugative and conjugative plasmids. *ori* = origin of replication, *tra* = transfer genes, *bom* = basis of mobility, *mob* = nicking protein synthesizing genes[45].

A complete conjugal apparatus is very large (e.g. RP II = 57 kb) and have *bom* site (ori^T) and/or ori^r and *mob* site (DNA nicking protein synthesis site recognizing *bom* site) and its handling in gene transfer experiments is very difficult. When the conjugal apparatus is incomplete naturally or is made short for their efficient use in cloning experiments, coresident plasmid(s) helps in conjugal transfer by coding conjugal gene transfer helping proteins. Based on their functions (code), these coresident plasmids are called conjugal plasmid (with *tra* genes), helper plasmid (with *mob* genes) and cargo plasmid (with *bom* or ori^T and/or ori^r genes) and DNA to be inserted/transformed. Cargo plasmids may be shuttle vector type or suicide vector type depending on the presence or absence of origin of replication ($ori^r/ori^v/ori$). Suicide vectors are of great help in transposon mutagenesis, gene replacement as well as integration of the vector into the recipient cyanobacterial chromosome.

Broad host range conjugal plasmid RP4 isolated from *Pseudomonas* (having genes for tetracycline, neomycin, kanamycin and ampicillin) of incompatibility group IncP and its derivatives pRL443 (without kanamycin resistance), R702 (with kanamycin resistance) and R751 (with trimethoprim resistance) are being used as conjugal plasmids[46]. Due to presence of *tra* genes these plasmids form sex pili and other surface components which help in physical contact of donor and recipient cells. Conjugal plasmid RP4 mobilizes derivatives of pBR322 to various gram negative bacteria if the vector contains the *bom* region (ori^T) of pBR322 and if the *trans* acting factors required for mobilization are provided by helper plasmids. Plasmids of other incompatability groups, e.g. W-incompatibility group plasmids S-a (Sm^r) and R7K (Sm^r) did not promote transfer. It is believed that capability of a cell to receive plasmid depends on outer cell membrane composition, while its maintenance depends on its resistance against host endonucleases e.g. restriction enzymes AvaI, AvaII, AvaIII and their isochizomers.

Helper plasmids either encode the Col E1 specific nicking protein that recognizes the *bom* site of Col E1 like plasmids, e.g. plasmids pDS4101 (ColK::Tn*1*), pGJ28 (Col D Kam^r), or carry a gene for methylase for evading the restriction by the recipient/host's endonucleases, e.g. pRL528, a mob^- derivative of Col k containing *Ava*I (methylase encoding genes from *Anabaena* PCC 7118) and *Eco*47II methylase. GGNCC sites have been modified by *Eco*47II methylase giving protection against the host's *Ava*II recognizing GGA/TCC site. Plasmid pRL449 (based on pACY184) and pRL518 (based on ColK) carries genes only for *Eco*47II methylase, whereas pRL530 (based on ColK)

carries genes for *Ava*I methylase[46]. Shuttle vectors with *Ava*III protection has also been developed[40].

Cargo plasmid is the actual transferred DNA and can be of shuttle vector type (replicate in the recipient due to presence of origin of replication, written as *ori* or ori^r) or suicide vector type (do not replicate in the recipient cell due to absence of ori^r). But in both conditions presence of *bom* site is essential. The cargo plasmids either have both *bom* site (ori^T) and replication site (ori^r) or only *bom* site. Plasmid pBR322 and its derivatives do not code for nicking protein but possess the *bom* site for the binding of nicking protein. This site is present between its origin of replication and PVII site (i.e. position 2207–2263). Therefore, pBR322 and many of its derivatives are being utilized as cargo plasmid but pBR328 and pUC7 from which *bom* site is deleted for increasing copy number cannot be used as cargo plasmids. For *Anabaena* PCC, *Nostoc*, *Fremyella* and *Plectonema*, shuttle vectors have already been constructed[47].

Construction of cyanobacterial shuttle vector pVW_1 and its derivatives[22,39]

Plasmid pDU1 of *Nostoc* PCC 7524 and plasmid pBR322 (Figure 2) of *E. coli* were used to construct shuttle vector type cargo plasmid for *Anabaena* and *Nostoc* (Figure 3) that was used to transform *E. coli* HB101. The chimaeric plasmid pVW1 thus obtained was 10.64 kb and had two disadvantages, viz. it has eight *Ava*II and one *Ava*I restriction site of pBR322 along with tetracycline (Tc) and ampicillin (Ap) resistance genes normally used as selectable markers. Tc^r cannot be used for photosynthetic cyanobacteria because of its light sensitive nature. The ampicillin marker mediated by β-lactamase also proved to be useless as it also protects the recipient strain.

Therefore, in pVW1 chloramphenicol (Cm) resistance gene of plasmid pBR328 is introduced after cutting it with *Sau*3AI. The fragment obtained possessed entire structural genes for chloramphenicol acetyl transferase. Plasmid pVW1 after having chloramphenicol resistance gene at *Bom*HI site it is designated as pVW1C but still it had the *Ava*II sites, which were eliminated by restriction enzyme *Ava*II. This reduces the potential problem of restriction of introduced plasmid by the endonucleases of the recipient cell. For avoiding religation of *Ava*II sites calf intestine alkaline phosphatase (CIAP) is used which removes the terminal phosphate by dephosphorylation and it is further digested with *Sau*96I, which restricts DNA at incompletely specified sequences (GGNCC), i.e. many sequences with different terminal sequences are formed, out of them only complementary fragments will recombine. Alternatively the restriction of shuttle vector into the

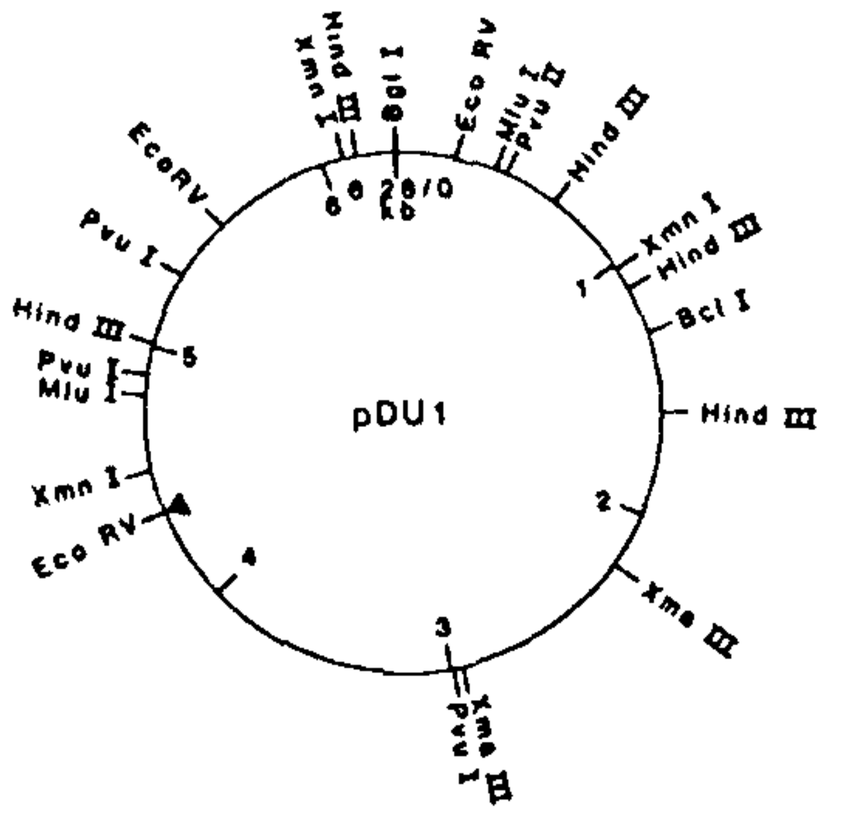

Figure 2. Restriction map of pDU1[22] and pBR322. A site of insertion of pBR322 in pVW1. The three sections of pBR322 are derived from Tn*3*, pMB1 and pSC101. The reading frame for beta lactamase (Ap^r = Ampicillin resistance) and three reading frames of proteins within the section derived from pSC101 are shown. Ori = origin of replication and Tc^r = tetracycline resistance.

recipient/host cyanobacteria can be achieved by premethylation of sites for *Sau*96I (including *Ava*II), *Ava*I and *Ava*III[40] and partially *Hae*III. Plasmid pRL1 thus obtained can be further treated to get its derivatives pRL5, pRL6, pRL8 (Figures 3 and 4). Plasmid pRL5 contains streptomycin (Sm) resistance gene from plasmid R300B, whereas pRL6 and pRL7 contain kanamycin (Km)/neomycin (Nm) resistance gene from Tn5. Plasmids pRL10 and pRL1 contain erythromycin resistance gene from pE194 in opposite orientations. Plasmid pRL8 is not being used because it regained *Ava*II site as cloning accident (Figure 4). Ampicillin resistance (Ap^r) has been shown to be expressed strongly by *Anabaena* sp. PCC 7120 exconjugants that have received β-lactamase gene of pBR322 on a shuttle vector. The conjugal efficiency of pRL1 to *Anabaena* M-131 was more than pVW1C, which may be due to lack of a large unwanted fragment of pBR322 in pRL1.

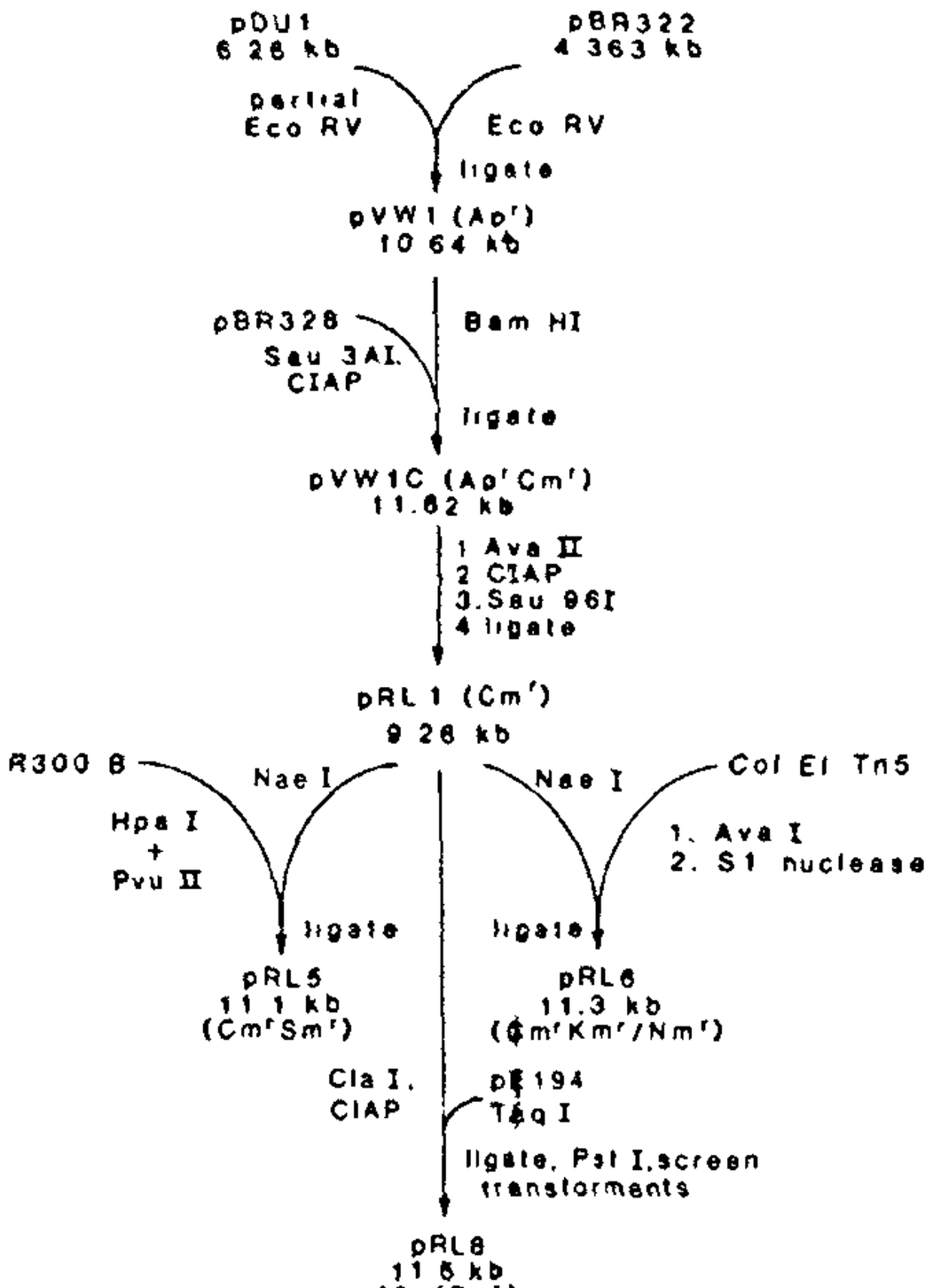

Figure 3. Construction of pRL1 and its derivatives pRL5, pRL6 and pRL8. CIAP = calf intestinal alkaline phosphatase[22].

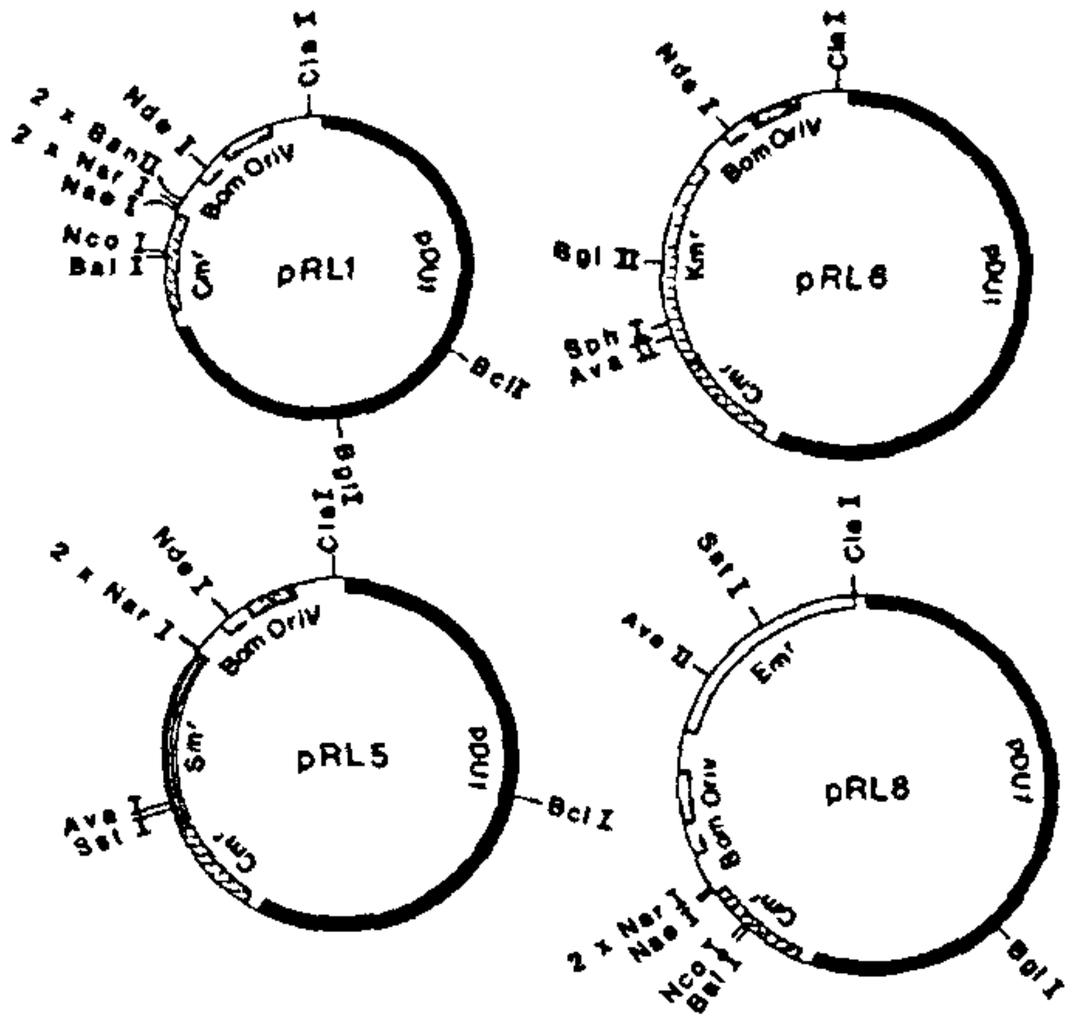

Figure 4. Maps of plasmid pRL1 and its derivatives pRL5, pRL6 and pRL8[22] showing potential cloning sites, the portions derived from pDU1 (solid bars) and pBR322 (lines), Cm^r fragment from pBR328 (cross hatched bars), the Sm^r fragment from R300B (lined bars), the Km^r fragment from Tn*5* (hatched bars) the Em^r fragment from pE194 (empty bars) and the positions of the origin of vegetative replication, *ori*V (stippled bars) and the *bom* region (arrow).

General methodology

Mobilisation of the shuttle vector into the recipient strain involves mixing of donor strain (biparental mating)/strains (triparental mating) having shuttle vector, conjugal plasmid and helper plasmid. Usually spot mating is preferred for testing new strain/new plasmid, while plate mating is preferred for finding rare exconjugants. The following steps are being adopted:

Step I–First of all cargo plasmid, e.g. pRL1, pRL5 or pRL8, is transferred to *E. coli* HB101 having the helper plasmid pGJ28 (Col D Km^r) by transformation, while pRL6 is transferred to *E.coli* HB101 having the helper plasmid pDS4101 (Col K::Tn*1*). Cargo and helper plasmid must be of two different incompatability groups so that they can replicate independently without interfering with each other and can avoid dilution effect of replication proteins.

Step II–The conjugal plasmid RP4 is transferred to *E. coli* J53. The two donor parents (one from step I and the other from step II) are allowed to grow overnight at 37°C in Luria broth with Km, Ap and Cm.

Step III–Such suspensions are left to mate at room temperature. During mating conjugative plasmid is transferred to the strain carrying the shuttle vector and the helper plasmid.

Step IV–One week old cyanobacterial cultures are used as third parent (recipient parent). The age of the recipient (target) cyanobacteria is not found to be critical. Mixing of the donor and the recipient was done on filter paper supported by cyanobacterial medium supplemented with 5% Luria broth and 1% agar (one day old).

In the presence of helper plasmid, the conjugative plasmid mobilizes the cargo plasmid from *E.coli* to the recipient cyanobacteria. Among three transferred plasmids, only cargo plasmid survives restriction and selection. Cells are allowed to grow on nonselective medium for about 24 hours, i.e. the optimal conditions for cyanobacterial growth expression of drug resistance. Same filter paper (means of convenience) was transferred on the selective medium for allowing the growth of exconjugants only. Selection of conjugated cyanobacteria was done against unmated control. It is advisable to use different dilutions of cyanobacteria on mating filter paper because, at high concentrations, nonmated cyanobacteria also grow on selection plate, probably due to synergistic effect.

Conjugal plasmid pRL443, a derivative of RP4 (Ap^r, Tet^r and Kn^s), and helper plasmid pRL528 (Cm^r) are

transferred into *E. coli* and stored as competent cells in Dr Golden's lab. Before going for conjugation, cargo plasmid with the recombinant gene is also transferred into these competent cells and then used as single donor parent. Helper plasmid pRL528 contains *mob* gene of Col K (which permits mobilization of plasmid with *bom* site), genes encoding the *Anabaena variabilis Ava*I methylase and *Eco* 47II methylase (which methylates at GGNCC and protects against *Ava*II, *Sau*96I and partially *Hae*III). Conjugal plasmid pRL443 is an RP4 derivative and lacks *Kan*^{r} gene. It provides *tra* genes responsible for formation of pili and other surface components necessary for conjugation to take place. Instead of performing spot or plate mating liquid mating can also be done. The exconjugants are plated on BG-11 plates having selected antibiotic(s).

Exconjugants are made *E. coli* free (axenic) by streaking/patching and restreaking/repatching followed by inoculation in liquid BG-11 medium with antibiotic(s). The presence of cargo plasmid in cyanobacterial exoconjugant is a proof for the success of conjugal transfer, which is done by isolation and characterization of the plasmid. It is better to do reconfirmation by transformational transfer of exconjugant isolated shuttle vector into *E. coli* and its reisolation and characterization.

Conclusion

The problem associated with direct gene transfer in filamentous cyanobacteria (like *Anabaena, Nostoc, Fremyella, Fischerella, Plectonema*) has been solved during the last decade through the development of the potentiality of conjugal gene transfer. It has opened the way for gene manipulation studies in other biotechnologically important filamentous cyanobacteria like *Spirulina* and *Westiellopsis*, provided one takes for surity of conjugal contact, avoidance of degradation of transferred DNA from host's (recipient's) restriction enzyme digestion and replication or integration of the transferred gene (plasmid) into the recipient cell. Conjugation will also be useful in transferring short peptide synthesizing genes such as genes for therapeutic proteins, insecticidal proteins, enzymes, etc. The advantage of cyanobacterial forms over higher plants in the 'biomolecular farming' is their photosynthetic ability, high growth rates and anoxygenic nitrogen fixation capability. The conjugal gene transfer holds the promise to achieve these objectives in a shorter span of time. However, some innovation in increasing the conjugal gene transfer efficiency needs to be studied for other biotechnologically important filamentous cyanobacteria which are not yet tried, e.g. *Spirulina*. The efficiency of gene transfer to cyanobacteria is fairly high and variable and depends on the type of strain as well as the type of DNA transferred. Many strains accept and express plasmid (e.g. pRL6) rarely or never. Plasmid pRL6 transfers well to *Anabaena* sp. M131, *Anabaena* sp. PCC 7120, *Anabaena* PCC 7118, *Nostoc* sp. MAC, *Nostoc* sp. strain ATCC 27896 (PCC 6310), *Nostoc* sp. strain 29133 (PCC 73102), *Nostoc* sp. strain 29150 (PCC 7107). According to Thiel, T. (personal communication) the efficiency of gene transfer for these strains is variable but fairly high (10^{-1}–10^{-5} per recipient cell). For *Anabaena* PCC 7120 it is about 10^{-4}–10^{-6}. In contrast, pDU1 based plasmids transfer poorly to *Anabaena variabilis* ATCC 29413 ($< 10^{-8}$ per recipient cell). Flores and Wolk[23] have found highest frequencies of recovery of presumptive exconjugants with *Nostoc* sp. strain ATCC 27896 ($1 \times 10^{-3} \times 10^{-3}$) for transfers involving pRL6 and pRL8. The generation time for exconjugants is different in autotrophic and facultative heterotrophic strains. For autotrophic strains in general generation time is 4–10 days for antibiotic-resistant colonies to appear, whereas for facultative heterotrophic strains it is 1.5–6 days. It also depends on the nature of the carbon source (glucose/fructose/sucrose). But if gene replacement by integration in chromosome is required, segregation of mutants and wild type copies may require several weeks. The factors which need attention are development of even broader host range plasmids, optimization of (more) efficient conjugal gene transfer conditions and development of cyanobacterial gene expression systems for high value metabolites other than that related with the understanding of basic molecular biology of cyanobacterial nitrogen fixation and photosynthesis. The work in future may be oriented to realize the above objectives in a profitable manner.

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RESEARCH ARTICLE

Climatic shifts over Mahanadi river basin

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The inter-annual variability and the long-term trends in the monsoon rainfall and in two derived climatic parameters, aridity index (I_a) and moisture index (I_m), have been examined for the Mahanadi basin using the rainfall and temperature data for the 80-year period (1901–80). The study shows that the basin has experienced a good number of deficit years during the last two decades of the study period. The yearly values of aridity and moisture indices show increase in the aridity conditions over the basin, with the semi-arid type climate in as many as five years during 1965–80. The trend analysis shows that the increasing and decreasing trends in the aridity index and moisture index respectively are statistically significant. The trend in the seasonal rainfall, though negative, is not statistically significant.

IN recent years there has been considerable interest in the study of climatic changes on global and regional scales because of their socioeconomic impacts. There is a vast literature on the subject; a critical review has been given by Ellsaesser *et al.*[1]. In the Indian context two recent studies are those of Pant *et al.*[2] and Rupa Kumar *et al.*[3]. In the latter study the authors found a decreasing trend in the monsoon rainfall of India for the period 1871–1984, over the area covering a major part of Madhya Pradesh, Orissa, Bihar and adjoining areas of Maharashtra, Uttar Pradesh and West Bengal. The trends are highly significant over eastern parts of Madhya Pradesh. Such decrease has adverse impact on national activities in which water has a major role. The present study is addressed to temporal variations in the climatic regime over the Mahanadi river basin on the basis of relevant meteorological data for the 80-year period (1901–80).