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Efficient Gene Transfer in *Synechococcus* sp. Strains PCC 7942 and PCC 6301 by Interspecies Conjugation and Chromosomal Recombination

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We developed a versatile, efficient genetic transfer method for *Synechococcus* sp. strains PCC 7942 and PCC 6301 that exceeds natural transformation efficiencies by orders of magnitude. As a test case, we complemented a histidine auxotroph and identified a *hisS* homolog of PCC 7942 as the complementing gene.

The cyanobacterium *Synechococcus* sp. strain PCC 7942 is naturally transformable. An endogenous plasmid can be used as the basis of autonomously replicating vectors. Alternatively, segments of the genome cloned into nonreplicating vectors will recombine with homologous DNA on the chromosome through events that predictably do or do not integrate heterologous vector sequences. However, the transformable phenotype is lost at a relatively high frequency (11); a mutant clone of desired phenotype may be untransformable and thus not an appropriate host for complementation by transformation with a library (18). The efficiency of transformation in the wild type, while suitable for engineering genes at specific sites, is not much higher than the reversion frequency of some point mutations. Successful uses of complementation in this organism have relied largely on phenotypes that provide strong selective pressure for the desired event and have often involved combining information about known restriction fragment size to bias library construction and improve the odds (5, 15, 18, 19). As research involving this organism becomes more sophisticated and the mutant phenotypes of interest may be more subtle, the need for an efficient gene transfer system becomes very important.

We combined the strategy of mobilizing DNA from *Escherichia coli* to the cyanobacterium (7) with use of the organism's efficient homologous recombination system (2, 5) to develop a very efficient means of generating or complementing PCC 7942 mutants. The strategy requires no special shuttle vectors, allows rapid recovery of the locus of interest, and applies to nontransformable isolates and to the closely related nontransformable *Synechococcus* sp. strain PCC 6301, the reference strain of the genus (16).

We used a histidine auxotroph, AMC254, which allowed easy identification of complemented clones. It was isolated by mutagenesis with nitrosoguanidine followed by two rounds of penicillin selection (8). AMC254 was grown in liquid or on solid modified BG-11 medium (3) supplemented with 10 to 100 μM histidine. Attempts to complement the auxotroph by addition of wild-type chromosomal DNA or a plasmid library of wild-type genomic fragments yielded no more colonies on BG-11 lacking histidine than those obtained by reversion of the auxotroph in the absence of DNA (data not shown). Subsequent tests indicated that the transformation efficiency of the mutant was approximately one-fifth that of the wild-type strain.

We used a mobilizable derivative of a PCC 7942 shuttle vector, pSG111M (Table 1), to work out conditions for triparental mating based on the procedure described by Elhai and Wolk (7). pSG111M was used to transform the *E. coli* cargo host (AM179). Overnight cultures of the conjugal strain (AM76) and transformed AM179 (100 μl of each) were washed twice with sterile deionized water and mixed with 200 μl of PCC 7942 cells (optical density at 750 nm of 0.4 to 0.5, washed twice with modified BG-11). The mixture was serially diluted, samples were plated on a mixture of modified BG-11 (3) and 5% LB (13) (vol/vol), with or without 7.5 μg of chloramphenicol per ml, and plates were incubated under standard growth conditions for PCC 7942 (3). Chloramphenicol-resistant transformants of PCC 7942 were obtained at near unity, with one colony forming on the antibiotic plate per two to seven PCC 7942 cells present in the mating (Table 2). No transformants were obtained when only AM76, but not the cargo strain, was present in the mating mixture.

We modified the gene transfer procedure to deliver a library of cloned DNA fragments in a nonreplicating vector, a kanamycin-resistant derivative of pBR322 (Table 1, pAM1153). *Synechococcus* chromosomal DNA was cleaved with *Sau*3A, and 2- to 4-kb fragments were cloned into the *Bam*HI site of pAM1153. The ligation products were used to transform AM179, and the pool of approximately 10,000 *E. coli* transformants was mixed with AM76 and AMC254 for mating. Kanamycin-resistant cyanobacterial colonies formed at a frequency of one per 300 to 500 AMC254-recipient cells (Table 2), and three colonies grew on medium without histidine (of 50,000 AMC254 recipients). No colonies formed in the absence of added histidine when the vector lacking an insert of PCC 7942 DNA was used as the donor DNA instead of the library or when either *E. coli* parent was omitted from the mating mixture.

Southern analysis confirmed the integration of vector sequences at different loci of the cyanobacterial genome in eight randomly selected kanamycin-resistant transconjugants (data not shown). The recombination event should produce a duplication at the site of insertion, and the duplicated region should be the size of the insert present in the transforming plasmid (6, 9). Therefore, it is straightforward to recover the integrated vector, along with a segment of flanking DNA, to identify the

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TABLE 1. Strains and plasmids used

Strain or plasmid	Description	Reference or source
Synechococcus sp. strains		
AMC254	Histidine auxotroph of *Synechococcus* sp. strain PCC 7942	This study
PCC 6301	Wild type	Laboratory collection
PCC 7942	Wild type	Laboratory collection
E. coli strains		
Conjugal strain AM76[a]	MRPO[RP4]	J. Shapiro, University of Chicago
Cargo host AM179[a]	HB101[pRL528]	7
Plasmids		
pAM717	pBR328 with a 2.8-kb *Bam*HI fragment from PCC 7942 (neutral site) which contains a unique *Xho*I site	4
pAM1146	pBR322 with the same PCC 7942 neutral site fragment as pAM717; contains the Ω cassette at the unique *Xho*I site of the PCC 7942 insert	This study
pAM1153	Km^r derivative of pBR322; Km^r gene from pUC-4K cloned into the *Pst*I site of pBR322 *bla* gene	This study
pAM1179	Derivative of pAM717 that contains Km^r gene from pSKS101 at the *Xho*I site of the *Synechococcus* insert	12a
pAM1214	*Eco*RV derivative plasmid rescued from complemented AMC254; contains most of pAM1153 and a 9.5-kb chromosomal segment from PCC 7942; complements AMC254	This study
pAM1217	*Nco*I-*Eco*RV deletion derivative of pAM1214; complements AMC254	This study
pAM1376	*Pvu*II derivative plasmid rescued from complemented AMC254; contains most of pAM1153 and a 0.7-kb chromosomal segment from PCC 7942; does not complement AMC254	This study
pBR322	Cloning vector (Ap^r Tc^r)	1
pRL528	Helper plasmid, pDS4101 derivative (Cm^r), ColK, *mob*, M.*Ava*I, M.*Eco*47II	7
pSG111M	Mobilizable *Synechococcus* shuttle vector; replicates in *E. coli* and contains the *bom* site	7
pSKS101	Source of Tn*5* Km^r cassette for pAM1179	17
pUC-4K	Carries Tn*903* Km^r GenBlock	Pharmacia
RP4	Conjugal plasmid (Ap^r Km^r Tc^r), IncP	J. Shapiro, University of Chicago

[a] Laboratory strain collection accession number.

integration locus (Fig. 1). Digestion of chromosomal DNA from His^+ transconjugants with either *Pvu*II or *Eco*RV and ligation of circularized fragments generated plasmids that contained chromosomal DNA from either side of the insertion site (data not shown). Plasmids recovered from two of the three transconjugants showed identical restriction patterns, and one appeared to be unrelated to these. The two identical *Eco*RV derivative plasmids (pAM1214) complemented AMC254, whereas the *Pvu*II derivative plasmids (pAM1367) did not. The *Eco*RV and *Pvu*II derivative plasmids from the third transconjugant did not complement AMC254, and we concluded that this transconjugant represented conjugation of an unrelated plasmid insert into a revertant cell.

The size of the insert in pAM1214 was decreased by digestion with various restriction enzymes to produce a series of plasmids with sequentially smaller inserts. Because the

TABLE 2. Efficiency of transfer of various donor DNA types to *Synechococcus* sp. strains PCC 7942 and PCC 6301 by conjugation and transformation

Recipient strain	Donor DNA	Transfer method	Integration/replication mode	Antibiotic selection	No. of transformants/recipient cell[a]
PCC 7942	pSG111M	Conjugation	Replicating plasmid	Chloramphenicol	5.6×10^{-1} 1.5×10^{-1}
	pAM1217	Conjugation	Single recombination	Kanamycin	3.5×10^{-3} 3.6×10^{-4}
	pAM1217	Transformation	Single recombination	Kanamycin	1.0×10^{-7}
	pAM1146	Conjugation	Double recombination	Spectinomycin	9.7×10^{-4}
	pAM1179	Transformation	Double recombination	Kanamycin	2.0×10^{-5}
	Library (2–4-kb inserts) in pAM1153	Conjugation	Single recombination	Kanamycin	1.8×10^{-3} 3.5×10^{-3}
	Library (0.6-kb inserts) in pAM1153	Conjugation	Single recombination	Kanamycin	3.4×10^{-3} 9.5×10^{-4}
PCC 6301	pSG111M	Conjugation	Replicating plasmid	Chloramphenicol	3.9×10^{-1}
	pAM1217	Conjugation	Single recombination	Kanamycin	7.3×10^{-4}
	pAM1217	Transformation	Single recombination	Kanamycin	0
	pAM1179	Transformation	Double recombination	Kanamycin	0

[a] When two numbers are shown for a given donor DNA, these values indicate the frequencies in two separate experiments.

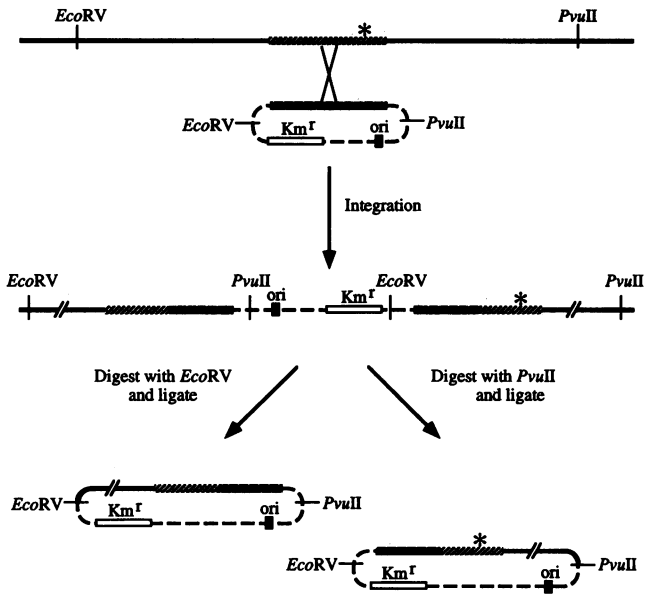

FIG. 1. Strategy for complementation and plasmid rescue in *Synechococcus* sp. strain PCC 7942. Recombination between the chromosome and a homologous segment of DNA cloned into a nonreplicating vector results in integration of the plasmid at this locus. Digestion of chromosomal DNA from the transformed strain with certain restriction enzymes (indicated) will release a segment of cyanobacterial DNA attached to the vector antibiotic resistance gene and origin of replication. Ligation and transformation of *E. coli* will retrieve the plasmid. Vector and chromosomal DNA segments are represented by different patterns (■, insert DNA; ——, chromosomal DNA; - - - -, vector DNA [pAM1153]; ▨, target DNA). Boxes represent the replication origin for *E. coli* (ori) and a kanamycin resistance gene (Km^r). An asterisk indicates a mutation in the chromosome.

original library contained inserts of 2 to 4 kb, the complementing segment should be within 4 kb of the junction with the vector. Figure 2 shows the results of complementation tests with the series of plasmids. These data narrowed the complementing region to a 0.76-kb segment between the *Xho*I and *Bam*HI sites. Nucleotide sequence analysis from pAM1217 (Fig. 2) showed the region to be part of a gene (*hisS*) whose predicted product is 49% similar to *E. coli* histidyl tRNA synthetase (Fig. 3). Restriction site analysis showed that the recovered *Pvu*II-derived plasmids (pAM1367), which did not complement AMC254, did not contain the segment that carries the *hisS* gene.

Additional conjugation experiments were performed with wild-type PCC 7942 to determine how the efficiency of conjugal gene transfer compared with transformation for different types of donor plasmids. Transformation by recombination with the chromosome in PCC 7942 is typically 100- to 1,000-fold higher for apparent double recombination events than for single recombinations; the type of recombination event recovered depends on the placement of the selectable marker within (double) or outside (single) the cloned segment that is homologous to the chromosome (9). A double recombination vector that targets a spectinomycin-streptomycin resistance cassette to the chromosome at a locus that we have termed a neutral site (4) was used in conjugation (pAM1146), while a similar vector that targets a kanamycin resistance cassette to the same site of the chromosome was used for transformation (pAM1179). The results in Table 2 confirmed that transformation efficiency was >100-fold higher when donor DNA was a substrate for double recombination than for single recombination (pAM1217 via transformation versus pAM1179). Surprisingly, this difference was not detectable when the donor DNA was introduced by conjugation (pAM1146 versus pAM1217 via conjugation). The *Synechococcus* reference strain PCC 6301, which is not transformable, produced frequencies of transconjugants equivalent to those produced by PCC 7942 (Table 2). Appropriate controls indicated that the resulting antibiotic-resistant colonies were genuine transconjugants (data not shown). Thus, PCC 6301, while incompetent for DNA uptake, is able to recombine with and incorporate DNA delivered by conjugation. Transfer of autonomously replicating RSF1010-derived plasmids to PCC 6301 by conjugation was previously demonstrated at a frequency of 2×10^{-6} to 6×10^{-6} (14). As expected from the close relationship of the two strains (10), PCC 7942 sequences recombined with the PCC 6301 chromo-

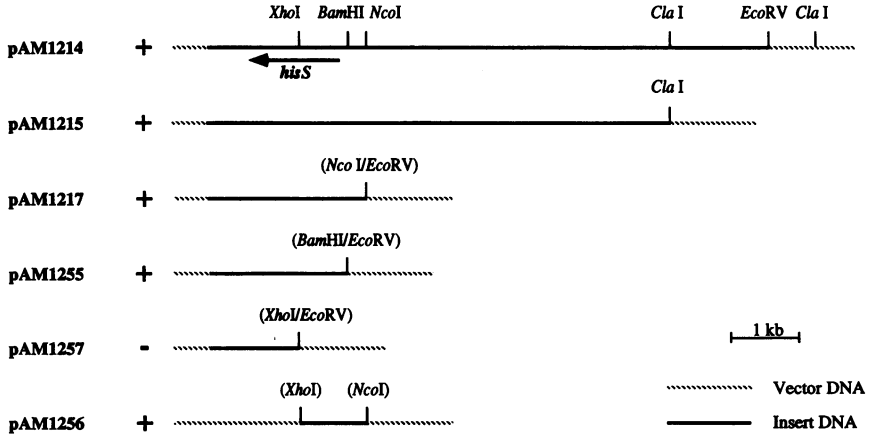

FIG. 2. Complementation assay of rescued plasmid and deletion derivatives. The segment of PCC 7942 DNA rescued from a His^+ AMC254 transconjugant is shown, with the position of the *hisS* open reading frame indicated by an arrow. Sites are indicated for those restriction enzymes used in creating deletion derivatives to define the complementing segment. Restriction enzyme names are shown in parentheses if these sites were destroyed in the cloning process. Each plasmid was tested by conjugation into AMC254 to assess its ability to complement the histidine auxotroph phenotype. Plus (complementation) and minus (no complementation) signs indicate the outcome of the test. Vector and chromosomal DNA segments are represented by different patterns as indicated in the key.

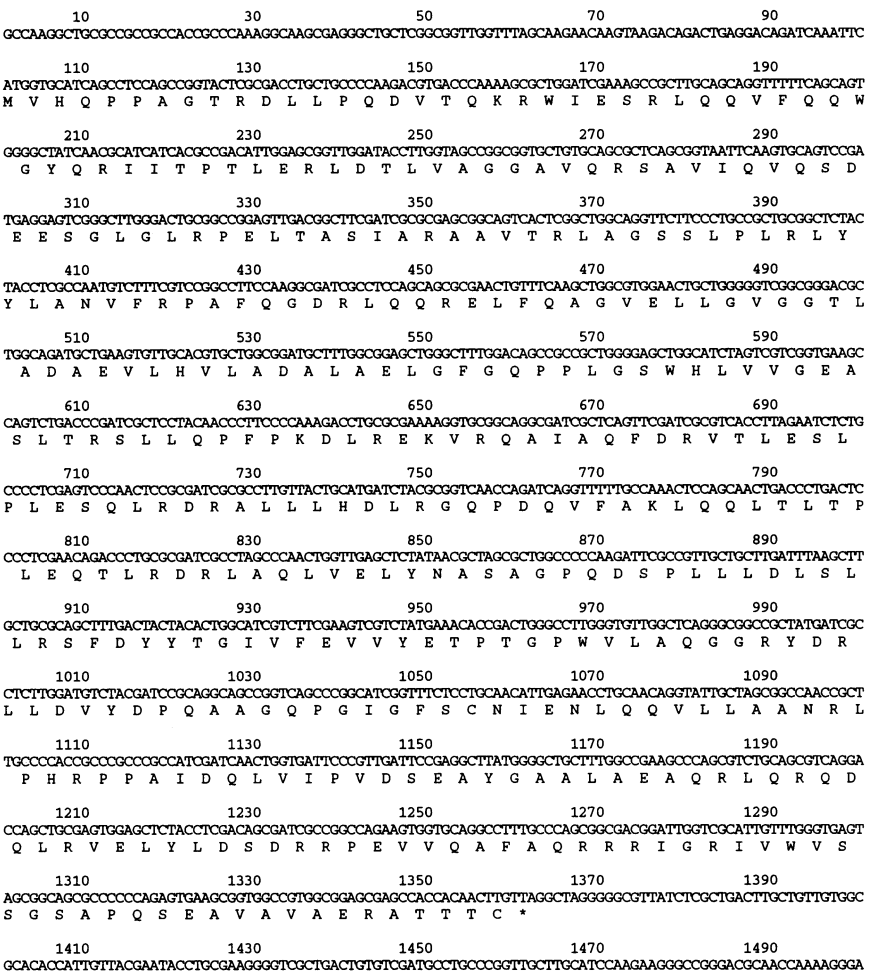

FIG. 3. Nucleotide and deduced amino acid sequences of the *hisS* gene from PCC 7942. Approximately 1.8 kb of nucleotide sequence was determined on both strands from pAM1217 (leftward from the *Bam*HI site indicated in Fig. 2). The deduced amino acid sequence is indicated by the single-letter code for the *hisS* gene.

some. This experimental protocol removes the necessity to switch from PCC 6301 to PCC 7942 for genetic analysis.

A conjugal library of small inserts of PCC 7942 DNA was prepared for creating insertion mutants by recombination with subgenic cloned fragments. Visible mutant phenotypes in the transconjugant pool included altered pigmentation and elongated cells (filamentous colonies). This is similar to the mutagenesis procedure recently described by Dolganov and Grossman using transformation but exceeded their efficiency by 3 orders of magnitude (300 to 800 transformants per 10^9 recipient cells, or 3×10^{-7} to 8×10^{-7} [6]). We have recently used this procedure to create mutants which show alterations in the circadian rhythm of bioluminescence (unpublished data) in a reporter strain (12).

The strategy of combining conjugal transfer from *E. coli* with recombination into the PCC 7942 or PCC 6301 chromosome removes the need for special vectors and renders all clones suitable hosts for recombinant libraries, regardless of transformability. The gene transfer efficiencies achieved by this procedure also allow such manipulations as introducing a reporter gene after mutagenesis of the host, which is not practical with efficiencies achieved by transformation protocols. Although transformation is easier, and is sufficient in many cases, the strategy presented here greatly expands the utility of *Synechococcus* sp. strains PCC 7942 and 6301 for sophisticated genetic manipulation that approaches the ease of working with *E. coli.*

Nucleotide sequence accession number. The sequence data reported have been assigned GenBank accession number L35476.

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