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Conjugative gene transfer in marine cyanobacteria: *Synechococcus* sp., *Synechocystis* sp. and *Pseudanabaena* sp.

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Summary. Versatility of gene transfer by transconjugation in marine cyanobacteria was demonstrated. In this study, seven different marine cyanobacteria were used as recipient cells. First, transconjugation was carried out using the mobilizable transposon (Tn*5*) carrying plasmid pSUP1021. Transconjugants were observed in all marine cyanobacteria tested. Second, the broad-host-range vector pKT230 (IncQ) was tested for transconjugation. pKT230 has been successfully transferred in a marine cyanobacterium *Synechococcus* sp. NKBG15041C, and replicated as an autonomous replicon without alteration in the restriction enzyme pattern. A maximum transfer efficiency of 5.2×10^{-4} transconjugants/recipient cell was observed, when mating was performed on agar plates containing low salinity (0.015 M NaCl) medium. This is the first study to demonstrate gene transfer in marine cyanobacteria via transconjugation.

Introduction

Marine biotechnology is expected to be a key technology in future bioindustry. Of the many resources in marine biotechnology, marine cyanobacteria play an important role, considering their high potential in producing various useful materials and also as promising candidates for the biological recycling of carbon dioxide. To achieve practical bioprocesses utilizing marine cyanobacteria, however, their productivity should be greatly enhanced. Therefore, molecular breeding of marine cyanobacteria will be essential.

Furthermore, marine cyanobacteria are an interesting research target in the field of molecular evolution. Marine cyanobacteria appeared on the earth about 2.7 billion years ago, and produce oxygen during photosynthesis. Since then, marine cyanobacteria have evolved independently from terrestrial organisms. Therefore, marine cyanobacteria can be considered as an independent genotypic group. However, little attention has been paid to the development of basic technology, such as gene manipulation, for molecular biological study of marine cyanobacteria. Gene manipulation in marine cyanobacteria has previously only been carried out using a naturally transformable strain, *Synechococcus* sp. PR6 (Stevens and Porter 1980; Essich et al. 1990). In addition, gene manipulation in freshwater strains was previously carried out with a few particular strains.

The researches based on gene transfer in freshwater strains utilized almost natural transformation with a particular strain (Grigorieva and Shestakov 1976; van den Hondel et al. 1980; Golden and Sherman 1984), while in some strains electroporation (Thiel and Poo 1989) and/or transconjugation (Wolk et al. 1984; Kreps et al. 1990) are available.

We have previously reported construction of a shuttle vector between marine *Synechococcus* sp. NKBG042902 and *Escherichia coli*, and transformation of this strain by electroporation (Matsunaga et al. 1990). Here, we demonstrate the versatility of conjugation as a method for gene transfer in several genera of marine cyanobacteria. Investigation of the potential for cloning vectors and genetic engineering systems of broad-host-range vector pKT230 (IncQ) was also carried out.

Materials and methods

Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* S17-1 (Simon et al. 1983) was used as the donor strain in which the chromosome derivative of RP4 was integrated. pSUP1021 is a mobilizable transposon (Tn*5*) carrier plasmid. *E. coli* strains were cultured in LB medium (10 g bacto-tryptone l^{-1}, 5 g bacto-yeast extract l^{-1} and 3 g NaCl l^{-1}; pH 7.4) at 37° C. All marine cyanobacteria used in this study were isolated from Japanese coastal seawater by the authors. Marine cyanobacteria were grown in marine BG11 media (ATCC catalogue, medium no. 617, containing 0.5 M NaCl), under continuous illumination (50 μEinstein m^{-2} s^{-1}), at 30° C for 1–2 weeks in a reciprocating shaker. All the strains are both kanamycin (Km) and streptomycin (Sm) sensitive. *E. coli* cells carrying either pSUP1021 or pKT230 were cultured in LB medium in the presence of 25 μg Km ml^{-1}. Cyanobacteria transconjugants carrying either Tn*5* in their chromosome or pKT230 were grown in a marine BG11 liquid medium in the presence of 25 μg Km ml^{-1}.

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Table 1. Bacterial strains and plasmids

Strain	Genotype	Reference
Escherichia coli		
S17-1	Δres[a], mod$^+$, thi, pro, hsdR, recA, RP4 derivative integrated	Simon et al. (1983)
DH5α	supE44, lacU169, (φ80, lacZΔM15), hasR17, recA1, endA1 gyrA96, thi-1, relA1	
Synechococcus sp.		
NKBG042902	Km^s, Sm^s	Matsunaga et al. (1990)
NKBG15041C	Km^s, Sm^s	This work
NKBG15031C	Km^s, Sm^s	This work
NKBG040606B	Km^s, Sm^s	This work
NKBG040607	Km^s, Sm^s	This work
Synechocystis sp.		
7a	Km^s, Sm^s	This work
Pseudanabaena sp.		
NKBG040605C	Km^s, Sm^s	This work
Plasmid	**Marker**	**Reference**
pSUP1021	Km, Cm, Tc	Simon et al. (1986)
pKT230	Km, Sm	Bagdasarian et al. (1981)

Km^s, kanamycin sensitive; Sm^s, streptomycin sensitive; Cm, chloramphenicol; Tc, tetracycline
[a] mod and res encode *Eco*P1 and *Eco*P15 R-M (restriction and modification) system genes, respectively

Analysis of genomic and plasmid DNA. Genomic and plasmid DNA preparation from cyanobacteria was carried out according to standard procedures (Porter 1988). DNA hybridization experiments were carried out using the non-isotopic labelling system [Digoxigenin-11-d uridine triphosphate (UTP), anti-digoxigenin enzyme-linked immunosorbent assay (ELISA)] provided by Boehringer (Mannheim, FRG). The *Xho*I restriction fragment of transposon Tn*5* and plasmid pKT230 were used as probe DNAs.

Bacterial mating and transformation. E. coli S17-1 cells were transformed with either pSUP1021 or pKT230. Colonies were collected, washed with sterilized water and resuspended again in sterilized water, and used for transconjugation after appropriate dilution. Mid-log-phase cyanobacterial cells were centrifuged (5000 *g*, 10 min, 25° C), washed twice with marine BG11 medium, and resuspended in the same medium at 1×10^9 cells ml^{-1}. *E. coli* S17-1/pSUP1021 or pKT230 suspension (100 µl) was added to 100 µl cyanobacterial suspension, mixed well and spotted onto a marine BG11 agar plate. Mating was carried out for 48 h under continuous illumination at 30° C. Cells were then removed from the agar plate and resuspended in marine BG11 medium. After appropriate dilution, 1 ml of this suspension was mixed with 5 ml of 0.5% top agar, and overlayed onto marine BG11 agar plates containing 25 µg ml^{-1} of Km or Sm. If the selection was carried out with liquid medium, mated cells were used to inoculate 10 ml marine BG11 medium containing 25 µg Km ml^{-1}. The cells were cultivated under continuous illumination at 30° C.

The ratio of *E. coli* to cyanobacterial cells used during conjugation (mating ratio) was varied by changing the donor cell (*E. coli* S17-1 with pSUP1021 or pKT230) concentration. Mating was also carried out using BG11 agar plates containing 0.015 M NaCl (low salinity).

The transformation of *E. coli* was carried out using competent cells stored at −70° C. The transformation with plasmid DNA was done according to Hanahan (1985).

Results

Transconjugation of pSUP1021(Tn5) toward marine cyanobacteria

We first investigated the versatility of transconjugation, and therefore chose plasmid pSUP1021. In this plasmid, the RP4-specific Mob site, which includes the origin of transfer, was cloned (Simon et al. 1983). pSUP1021 also harbours Tn*5*. Since this plasmid has a replicon of pACYC 184 only being replicated in the *Enterobacteriaceae*, transconjugants can be selected as Tn*5* transposon mutants, which are kanamycin resistant (Km^r) cells. We can, therefore, evaluate the potential of the application of gene transfer by transconjugation for marine cyanobacteria without considering the replication origin of the plasmid. After appropriate mating procedures (see Materials and methods), transconjugants were selected in a liquid medium supplemented with Km. Km^r cells were observed in all seven strains of marine cyanobacteria tested. Km^r cells were never observed in control mating experiments using *E. coli* DH5α cells, unable to perform transconjugation, harbouring pSUP1021. Therefore these Km^r cyanobacterial cells were not the results of the co-existence of marine cyanobacteria with Km^r *E. coli* cells.

We previously checked the possibility of natural transformation of marine cyanobacteria used in this study. However, these seven strains do not possess detectable transformation ability. Therefore, the effect of plasmids that might be exposed to the donor by cell lysis would be negligible for the observation of Km^r cells. However, using agar plates containing Km, no Km^r colonies were observed.

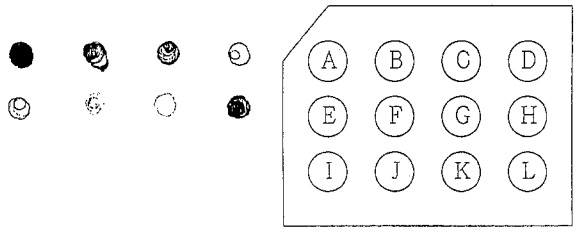

Fig. 1. Dot-blot hybridization analysis of the transconjugants. The Tn*5* fragment purified from pSUP1021 was used as a probe. Each sample (A–L) was spotted onto the filter as shown in the *right half* of the figure. The *first line* in the *left half* of the figure corresponds to *A–D* in the *right half* of figure, the *second line* corresponds to *E–H*, and third line, where no positive signal was observed, corresponds to I–L: *A*, 100 ng native pSUP1021 purified from *Escherichia coli*; *B*, 1000 ng total DNA from transconjugant strain NKBG15041C; *C*, 1000 ng total DNA from transconjugant strain NKBG15031C; *D*, 1000 ng total DNA from transconjugant strain 7a; *E*, 1000 ng total DNA from transconjugant strain NKBG042902; *F*, 1000 ng total DNA from transconjugant strain NKBG040605C; *G*, 1000 ng total DNA from transconjugant strain NKBG040606B; *H*, 1000 ng total DNA from transconjugant strain NKBG040607; *I*, 1000 ng total DNA from wild-type strain NKBG15041C; *J*, 1000 ng total DNA from wild-type strain NKBG042902; *K*, 1000 ng total DNA from wild-type strain NKBG040606B; *L*, 1000 ng total DNA from wild-type strain NKBG040607

Figure 1 shows the dot-blot hybridization of each of the Km^r marine cyanobacteria with the *Xho*I fragment of Tn*5*. All the Km^r cells hybridized with the Tn*5* probe. These results suggested that pSUP1021 was successfully transferred into all marine cyanobacteria tested.

Conjugative transfer of pKT230 into marine Synechococcus sp. NKBG15041C

pKT230, a broad-host-range vector (IncQ) plasmid was used as a transconjugation vector for the marine cyanobacteria to investigate whether a non-cyanobacterial plasmid can be replicated and maintained as an autonomous replicon in marine cyanobacteria. As recipient cells, a marine cyanobacterium *Synechococcus* sp. NKBG15041C, which had a high growth rate, was used. In this experiment, no Km^r colonies appeared on the agar plate containing Km, so selection was also carried out using liquid medium. As well as pSUP1021, Km^r cells were observed. Since pKT230 also encodes Sm^r, selection was also carried out using Sm. Although Km^r colonies were never observed on Km agar plates, Sm^r colonies were observed on Sm agar plates.

Figure 2 shows Southern hybridization analysis of Km^r cells probed with pKT230. The strong hybridization band suggests that pKT230 was transferred into this strain and autonomously replicated as an extrachromosomal plasmid.

The effect of mating ratio and salinity of the mating medium on the transconjugation efficiency was investigated by plating transconjugants onto Sm agar plates (Table 2). Transconjugation efficiency also increased with increase in the mating ratio. The transconjugation efficiency was 20 times higher when matings were carried out using low (0.015 M) salinity agar plates. The highest transconjugation efficiency obtained was 5.2×10^{-4}.

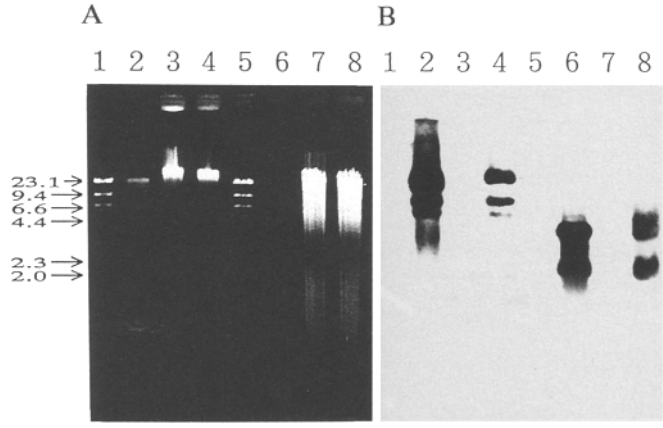

Fig. 2A, B. Southern hybridization analysis of transconjugant: *lane 1*, *Hin*dIII digests; *lane 2*, native pKT230 purified from *E. coli*; *lane 3*, total DNA from wild-type; *lane 4*, total DNA from transconjugant; *lane 5*, *Hin*dIII digests; *lane 6*, native pKT230 purified from *E. coli* digested by *Pst*I; *lane 7*, total DNA from wild-type digested by *Pst*I; *8*, total DNA from transconjugant digested by *Pst*I. **A** Transconjugant stained by ethidium bromide. **B** Southern hybridization analysis of transconjugant. The vertical numbers represent relative molecular weight markers in kbp

Table 2. Effect of mating ratio and salinity on efficiency of transconjugation

Mating ratio[a]	Transconjugation efficiency[b]	
	0.015 M NaCl	0.5 M NaCl
10: 1	6.0×10^{-6}	—
1: 1	6.6×10^{-5}	3.1×10^{-6}
1:10	5.2×10^{-4}	2.6×10^{-5}

[a] Cyanobacteria: *E. coli*
[b] Transconjugation efficiency is expressed as transconjugants/recipient

Plasmids from transconjugants harbouring pKT230 were extracted and transformed back into *E. coli*. pKT230 recovered from *Synechococcus* sp. NKBG15041c was able to transform *E. coli* DH5α. Restriction enzyme analysis of pKT230 from *E. coli* and originally from *Synechococcus* transconjugants showed no significant structural alterations (Fig. 3). Therefore, pKT230 was maintained in NKBG15041c as an autonomous replicon without significant structural modification.

Discussion

Gene manipulation in marine cyanobacteria is only available using *Synechococcus* PR6, a naturally transformable strain. In this study, we showed that transconjugation is a versatile method for gene transfer in marine cyanobacteria. The broad-host-range vector

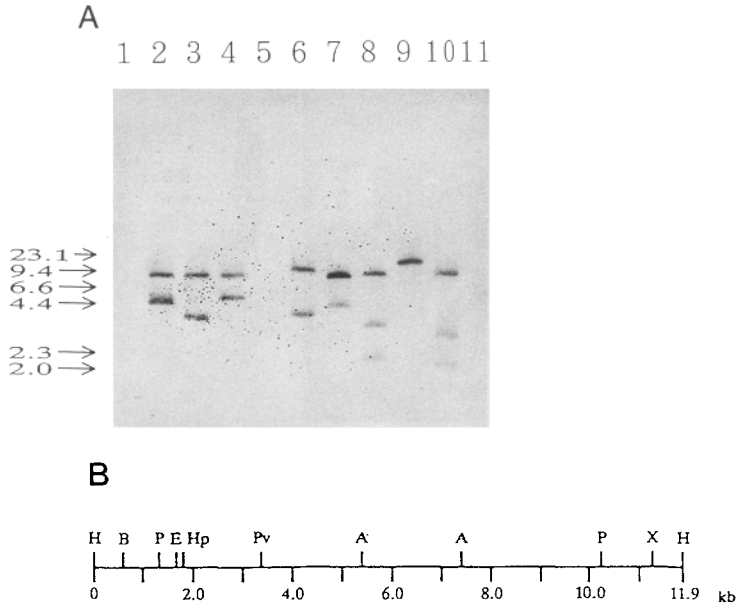

Fig. 3. A Restriction analysis of pKT230 after conjugation to *Synechococcus* NKBG15041C. pKT230 purified from kanomycin-resistant colony of *E. coli* strain DH5 obtained by transformation with DNA extracted from the *Synechococcus* transconjugant: *Lane 1*, *Hin*dIII digests; *lane 2*, pKT230 purified from DH5 digested by *Pst*I; lane 3, pKT230 purified from DH5 digested by *Pst*I and *Bam*HI; *lane 4*, pKT230 purified from DH5 digested by *Hin*dIII and *Pvu*II; *lane 5*, none; *lane 6*, pKT230 purified from DH5 digested by *Xho*I and *Eco*RI; *lane 7*, pKT230 purified from DH5 digested by *Acc*I; *lane 8*, pKT230 purified from DH5 digested by *Pvu*II, *Hpa*I and *Hin*dIII; *lane 9*, pKT230 purified from DH5 digested by *Hpa*I; *lane 10*, pKT230 purified from DH5 digested by *Eco*RI, *Xho*I and *Pst*I; *lane 11*, *Hin*dIII digests. **B** Restriction map of pKT230. Restriction sites are: *A*, *Acc*I; *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dIII; *Hp*, *Hpa*I; *P*, *Pst*I; *Pv*, *Pvu*II; *X*, *Xho*I. The vertical numbers represent relative molecular weight markers in kbp

pKT230 has also been transferred and this vector may also be used in further gene manipulation studies. This is the first study to demonstrate gene transfer in marine cyanobacteria via transconjugation. Furthermore, this research shows that transconjugation, which has been only investigated in terrestrial microorganisms, can function for marine microorganisms, and that the IncQ plasmid can also replicate in marine microorganisms. These results suggest the hitherto unknown existence of a possible gene transfer system in the marine environment.

The dot-blot analysis of Km^r cells resulting from the mating between donor cells with pSUP1021 and marine cyanobacteria (Fig. 1) revealed that these cells harbour Tn*5*. Although the same amounts of DNA were applied for analysis, the hybridization level was not same among the transconjugants. Due to the possible instability of the integrated Tn*5* and also the low viability of the transconjugant, the population of cells without Tn*5* might increase during cultivation, consequently resulting in a difference in hybridization level. The stability of transconjugants possessing Tn*5* is currently being investigated.

Transconjugants harbouring either Tn*5* or pKT230 could not be selected using agar plate medium containing 25 μg Km ml^{-1}, since no colony formation was observed. At Km concentrations lower than 25 μg ml^{-1} (5 and 10 μg ml^{-1}), even wild-type cells can grow and form colonies, so that selection was not possible using agar plates containing lower concentrations of Km. In this experiment, we concentrated on selecting transconjugants using a liquid medium containing 25 μg Km ml^{-1}, which resulted in significant differences in growth between control experiments and transconjugants in all strains tested.

Transconjugants that possess Tn*5* in their chromosome cannot form a single colony on the agar plate with 25 μg Km ml^{-1}, even after selection in liquid medium, whereas *Synechococcus* sp. NKBG15041C with pKT230 can form colonies on an agar plate with 25 μg Km ml^{-1}, after successive cultivations in liquid medium (results not shown). We believe that liquid medium is more suitable for cultivation of these marine strains than agar plate medium, and that cell viability and also foreign gene expression, such as antibiotic resistance, might be higher when cells are grown in liquid medium. Therefore, direct selection using Km agar plates cannot be applied to this system. Although the copy number of pKT230 in the cell was not high (considering the band observed in Fig. 2, lane 4 and 8), the gene dosage effect may result in the difference in Km^r colony formation in agar plates between transconjugants harbouring pKT230 and Tn*5*.

The difference in the expressed Km and Sm antibiotic resistance may be due to the expression level of the corresponding gene in the marine cyanobacteria.

If mating was carried out in medium with low salinity (0.015 M), transconjugation efficiency was drastically increased. It has been reported that *E. coli* enters a viable but non-culturable state under certain environmental conditions (Colwell et al. 1985; Xu et al. 1982), and high salinity appears to be one factor in this phenomenon. Lower transconjugation efficiency observed at high salinity may due to the lower activity of *E. coli* cells. We have carried out a preliminary experiment to investigate the effect of salinity on transconjugation between *E. coli* S17-1/pKT230 as the donor strain and *E. coli* HB101/pBR322 as the recipient strain. Transconjugation efficiency at low salinity was ten- to 20-fold higher than that carried out at high salinity. The impact of salinity on transconjugation was similar in these two cases, *E. coli* vs marine cyanobacteria and *E. coli* vs *E. coli*. Although in this experiment using *E. coli* as recipient cells, we cannot separate the effect of salinity on donor cells and recipient cells, the effect of salinity on *E. coli* transconjugation ability might dominate the transconjugation efficiency.

Many researches have been carried out on cyanobacterial membrane structure alteration due to salinity changes (Blumwald et al. 1983; Lefort-Tran et al. 1988;

Molitor et al. 1990; Huflejt et al. 1990). *Synechococcus* sp. NKBG 15041C cells were precultured in medium containing 0.5 M NaCl. Subsequent mating on 0.015 M NaCl agar for 48 h may induce a change in the cyanobacterial cell membrane, and consequently the physiologial properties of the membrane such as barrier functions and transport. Therefore, a salinity change may alter the membrane characterization, and consequently increase the transconjugation efficiency. Hence, the higher transconjugation efficiency observed under low-salinity mating conditions could be due to both an increase in *E. coli* activity and possible alteration of the cyanobacterial membrane. Since this is also the first research in which *E. coli* transconjugation with marine microorganisms has been demonstrated, further investigation is necessary for a full understanding of the salinity effect transconjugation between *E. coli* and other marine microorganisms.

A broad-host-range vector plasmid pKT230 was found to be maintained as an automonous replicon in a marine cyanobacterium, *Synechococcus* sp. NKBG15041C. To evaluate the possibility of pKT230 as the vector plasmid for *E. coli* and *Synechococcus* we checked that this plasmid recovered from *Synechococcus* transconjugants can be transformed in *E. coli* without significant structural change. This result suggests that this vector plasmid can be useful for further genetic analysis in marine *Synechococcus* spp. We are currently investigating the conjugal transfer of pKT230 to other marine cyanobacteria and the stability and maintenance of transferred plasmids in marine cyanobacteria.

In conclusion, gene transfer via transconjugation is a very promising method for marine cyanobacterial gene manipulation. The results presented here would contribute to the development of novel host-vector systems in industrially useful marine cyanobacteria.

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