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ORIGINAL PAPER

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# Gene transfer and manipulation in the thermophilic cyanobacterium *Synechococcus elongatus*

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**Abstract** DNA can be introduced into the thermophilic cyanobacterium *Synechococcus elongatus* by electroporation or conjugation. Its genome can be readily manipulated through integrative transformation or by using promiscuous RSF1010-derived plasmids that can be transferred unaltered between *Escherichia coli* and *Synechococcus elongatus*. These vectors can therefore be used for in vivo studies of cyanobacterial proteins in both mesophilic and thermophilic cyanobacterial backgrounds. As a preliminary step towards the analysis of structure-function relationships of photosystem I (PSI) from this thermophile, the genes encoding the PSI subunits PsaF, PsaL, and PsaK were inactivated and shown to be non-essential in *S. elongatus*. In addition, PSI reaction centres were extracted from a *psaL*$^-$ strain exclusively as monomeric complexes.

**Key words** Cyanobacteria · Integrative transformation Mutagenesis · Photosystem I · Replicative vectors

## Introduction

Cyanobacteria are the only group of phototrophic bacteria capable of performing oxygen-evolving photosynthesis using two photosynthetic reaction centres. Their electron transport chain is very similar to that of higher plants and this property was the prime incentive for earlier investigations of cyanobacterial genetics (Buzby et al. 1985; Vermaas et al. 1988; Vermaas 1994). Currently the genetic analysis of the photosynthetic apparatus is most frequently performed in the cyanobacteria *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002, since these strains possess a natural DNA uptake system and are able to grow heterotrophically (see for instance Barry et al. 1994; Golbeck 1994, for recent progress on photosystems I and II).

Thermophilic strains with optimal growth temperatures above 45° C are found among several cyanobacterial subdivisions (Rippka et al. 1979; Castenholz et al. 1989) and appear to be obligate photoautotrophs. Although they have so far proven to be refractory to transformation, several of these species are very popular among protein chemists as a source of easily purified and remarkably stable protein complexes. This is especially true with respect to the photosynthetic reaction centres; thus photosystem I (PSI) from the thermophilic strain *Synechococcus elongatus* has been successfully crystallised (Krauss et al. 1993). As a step towards the analysis of structure-function relationships of this PSI reaction centre, which has been comprehensively analysed with respect to its subunit composition and primary structure (Mühlenhoff et al. 1993; Golbeck 1994), we have initiated gene manipulation in this organism. In this context we report that DNA can be introduced into *Syn. elongatus* by electroporation or conjugation and that the genome of this thermophile can be readily manipulated through integrative transformation or with the aid of promiscuous plasmids. In addition, we report the analysis of the first PSI deletion mutants from this thermophilic strain.

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## Materials and methods

### Strains and culturing conditions

*Synechococcus elongatus* (Yamaoka et al. 1978), obtained from the Technische Universität Berlin, FRG, was colony purified after

treatment with cycloheximide, kanamycin and ampicillin according to Rippka et al. (1981). Cells were grown at 45° C under continuous illumination (1.5 to 5 mW/cm$^2$) on 1.2% agar plates in medium DTN, which corresponds to medium D of Castenholz (1988), supplemented with 0.8 mM $Na_2S_2O_3$, 1.6 mM $Na_2SO_3$, 0.2 mM $NH_4Cl$, 8 mM Tricine and 10 mM $NaHCO_3$. The pH of the medium was adjusted to 7.65 prior to sterilisation. Liquid cultures were grown in closed flasks under the same conditions and supplemented with air enriched with 1% $CO_2$ if culture volumes exceeded 5 ml. For selection and maintenance of transformants, liquid cultures are routinely supplemented with 40 μg/ml kanamycin [Km], 5 μg/ml chloramphenicol [Cm], 2 μg/ml streptomycin [Sm], or 20 μg/ml spectinomycin [Sp]. Agar plates are routinely supplemented with 25 μg/ml Km, 2.5 μg/ml Cm, 2 μg/ml Sm, or 10 μg/ml Sp. Strains are stored at −80° C in a small volume of DTN as recommended (Castenholz 1988). The addition of protectants such as glycerol or DMSO has deleterious effects. The strain dies rapidly when stored at 4° C.

DNA manipulations were performed in the *E. coli* strain XL1 blue by standard techniques (Sambrook et al. 1989). RSF1010-derived plasmids pFC1 (Mermet-Bouvier and Chauvat 1994) and pSB2T (Marraccini et al. 1993) were isolated from *E. coli* strain HB101 prior to electroporation or introduced into *E. coli* CM404 harbouring pRK2013 (Powell et al. 1989) prior to conjugative transfer to *Syn. elongatus* (Mermet-Bouvier et al. 1993).

### Transformation

For electroporation, cells were grown to $OD_{750}$ of 0.5 (approx. $6 \times 10^8$ cells/ml), washed once in the same volume of 2 mM Tricine, 2 mM EDTA pH 8.0, twice with double-distilled water and resuspended in water at 10-fold concentration. A 40-μl aliquot of the suspension was mixed with 4 μl of a 1 μg/μl DNA solution, chilled on ice for 2 min and electroporated with a single pulse with a time constant of 5 ms at field strengths of 4 to 12 kV as described by Thiel and Poo (1989). Pulsed cells were rapidly transferred to fresh DTN medium and were allowed to recover for 24 h prior to antibiotic challenge in liquid culture, gradually raising the concentrations of antibiotics to the required levels over a period of 5 days. Resistant cells appeared within 2 to 3 weeks and were colony isolated on solid media. In cases where integrative cartridges of donor DNA were used a second round of replating was performed in order to ensure complete segregation of the mutations. Conjugation was performed by the biparental mating procedure of Mermet-Bouvier et al. (1993). A cyanobacterial culture was grown to $OD_{750}$ of 0.5–1 and mixed in DTN + 5% LB with the same amount of an overnight culture of *E. coli* CM404 harbouring pFC1 or pSB2T. The mix was incuated at 30° C for 20 h under illumination (1.5 mW/cm$^2$). Aliquots (0.1 ml) were diluted in DTN medium and incubated for 24 h at 45° C to allow the cyanobacteria to recover and to ensure effective killing of the *E. coli* cells. The cyanobacteria were then transferred to a fresh culture and transformants were selected as described above.

### DNA and plasmid analysis

Genomic DNA and plasmids from *Syn. elongatus* were isolated from saturated 25-ml cultures essentially according to Porter (1988). Effective cell lysis required incubation with 2 mg/ml lysozyme at 48° C for at least 30 min. Southern blot analysis was carried out using standard hybridization protocols and biotinylated DNA probes labeled by random priming. The membranes were subsequently incubated with avidin linked to horseradish peroxidase and developed by luminol detection (Amersham) essentially as described previously (Pollard-Knight et al. 1990). Plasmids were re-transferred into *E. coli* strains XL1 blue, ER 1565 and ER 1647 by standard transformation techniques.

### PSI preparation and analysis

PSI was extracted from photosystem II-depleted membranes using 1% sulfobetain 12, essentially according to Schatz and Witt (1984) and purified by sucrose gradient centrifugation followed by ion-exchange chromatography on Q-Sepharose (Pharmacia) essentially as previously described (Rögner et al. 1990; Jekow et al. 1995). Molecular mass determinations were carried out by gel filtration chromatography on Sepharose CL-6B (Pharmacia) by reference to elution volumes of marker proteins as described previously (Rögner et al. 1990). SDS-polyacrylamide gel electrophoresis was performed on 17.5% acrylamide slab gels as described in Schägger and Jagow (1987) and stained with silver according to Blum et al. (1987).

## Results

### Construction of DNA cartridges suitable for assaying for integrative transformation in *Syn. elongatus*

Interruption of protein-coding regions of the gene of interest by insertion of antibiotic resistance genes provides a powerful tool for the genetic analysis of mesophilic cyanobacteria (for review see Haselkorn 1991; Thiel 1994). Upon transfer of such DNA cartridges into these hosts, homologous recombination occuring on both sides of the marker gene leads to its integration within the target gene, which consequently becomes inactivated. Gene inactivation has been used to show that several of the genes encoding PSI subunits are not essential for photosynthesis in mesophilic cyanobacteria (Golbeck 1994). Although not proven, it is reasonable to assume that these genes are also non-essential for thermophilic cyanobacteria such as *Syn. elongatus*, and should thus be ideal targets for gene inactivation in this obligate photoautotroph. In addition, since the loci disrupted in such mutants can be characterized with precision, an integrative transformation assay appeared to be ideal for testing gene transfer into this thermophilic bacteria. Therefore, antibiotic resistance DNA cartridges were constructed using genomic fragments carrying the genes *psaK* (EMBL accession no. X63764), *psaF* (X63765) and *psaL* (X63763) from *Syn. elongatus* (Mühlenhoff et al. 1993). The genomic fragments were cloned into the *E. coli* plasmids pBSCM13$^+$ (*psaK*) or pUC18 (*psaF*, *psaL*), and the coding regions of the respective genes coding for PSI subunits were either interrupted (*psaK* and *psaF*) or partially deleted (*psaL*) upon insertion of the antibiotic resistance genes, as outlined in Fig. 1.

### Transfer of integrative DNA using electroporation

The *E. coli* plasmids carrying PSI genes interrupted by an antibiotic resistance gene were used as donor DNA in a series of attempts to transform *Syn. elongatus*. The possibility that a natural DNA uptake system exists in *Syn. elongatus* was ruled out since the established

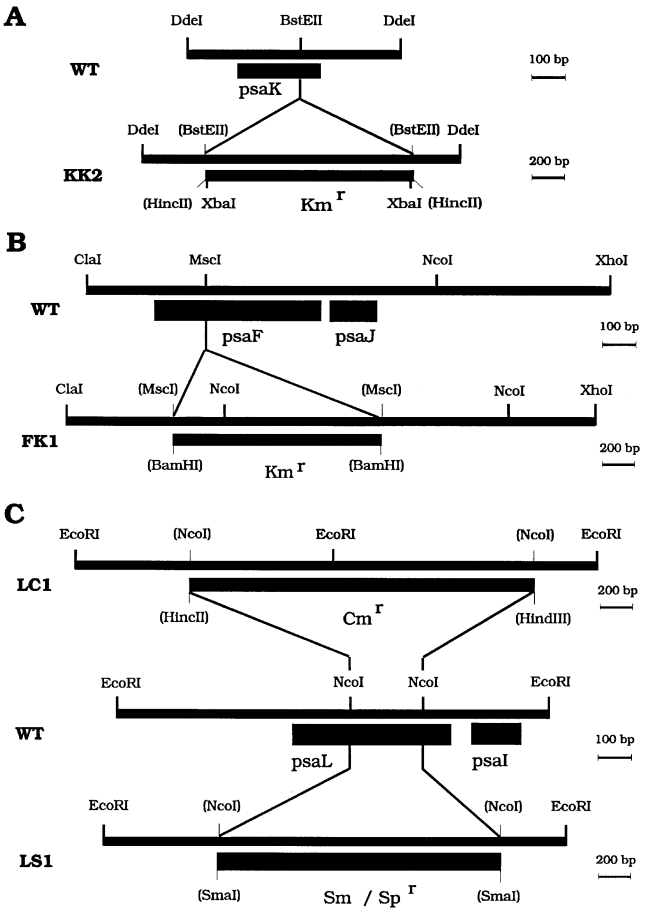


**Fig. 1A–C** Construction of integrative vectors for insertional inactivation of the *psaK*, *psaF*, and *psaL* genes in *Syn. elongatus*. For DNA sequences of the PSI genes see Mühlenhoff et al. (1993). Antibiotic marker genes were obtaned from pRL161 ($Km^r$; Elhai and Wolk 1989), pBR325 ($Cm^r$), and pHP45Ω ($Sm^r/Sp^r$; Prentki and Krisch 1984). Restriction sites in parentheses were lost during construction. For cloning, the DNA fragments were inserted into the *E. coli* vectors pUC18 (*psaF* and *psaL*) or pBSCM13$^+$ (*psaK*). Approximate fragment sizes are indicated by the *bars*

protocols for the transformation of the naturally competent cyanobacteria *Synechococcus* sp. PCC 7002 (Buzby et al. 1983) and *Synechocystis* sp. PCC 6803 (Williams 1988) proved unsuccessful in *Syn. elongatus*. Similarly, chemical treatments commonly used to transform *E. coli* remained ineffective. Therefore DNA uptake by electroporation was tested since it has proved to be successful in several cyanobacterial strains (Thiel and Poo 1989; Chiang et al. 1992; Mermet-Bouvier et al. 1993). Field strengths of 4 to 12.5 kV at a time constant of 5 ms were investigated. Above 11 kV the number of survivors declines drastically with increasing field strength. Successful transformation to antibiotic resistance was repeatedly obtained between 8 and 12 kV, i.e. at fields strengths very similar to those required for the filamentous cyanobacterium *Anabaena* sp. M131 (Thiel and Poo 1989).

At present, we cannot precisely quantitate the frequency of electrotransformation since antibiotic resistant clones could not be selected directly on plates following electroporation. Instead, so far transformants can only be recovered if antibiotic selection is slowly applied to cells growing in liquid culture prior to plating (Materials and methods). Once resistance was established in liquid cultures, transformants could be isolated as single colonies on solid media and propagated with ease in selective media, either liquid or solid, even though they invariably grow a little more slowly in the presence of the drugs (not shown). Slow adaptation of freshly transformed cells to selective antibiotics is widely used for mesophilic cyanobacteria and requires just a little more time in the case of this thermophilic strain. Together with the slightly reduced growth rates observed for transformants cultivated in selective media, this phenomenon suggests that the expression of marker genes originating from mesophilic bacteria, or the activity of the corresponding enzymes, is less at the elevated temperatures required for cultivation of the thermophilic host. We favour the second hypothesis since promotors of genes from *Syn. elongatus* (Mühlenhoff et al. 1993) contain *E. coli*-like elements, similar to those found in mesophilic cyanobacteria which are known to transcribe *E. coli* promotors at high levels (Elhai 1993).

## Analysis of transformants

Southern blot analysis (Fig. 2) showed that the antibiotic resistance phenotypes of isolated clones of *Syn. elongatus* arose through integration of the corresponding marker genes into the cyanobacterial genome at a single site. A single band was observed for each transformant DNA, while no bands could be detected in wild-type DNA serving as a negative control. Therefore, these clones were true transformants, as expected, rather than the result of mutations induced by electroporation as decribed previously in the case of the filamentous cyanobacterium *Fremyella diplosiphon* (Bruns et al. 1989). It seems that electroporation is also slightly mutagenic in *Syn. elongatus* since a few $Km^r$ mutants were occasionally obtained following electroporation performed in the absence of DNA.

Total DNA of transformants was further analysed by Southern blotting to study the genomic organization of the genes *psaF*, *psaK*, and *psaL*, which were expected to be interrupted in the corresponding transformants FK1, KK2, LC1 and LS1 (Fig. 3). In case of the *psaK* locus, the 12-kb *Xba*I restriction fragment carrying the wild-type *psaK* gene is split into two fragments of 10 kb and 1.9 kb in DNA of strain KK2, where this gene has been interrupted by a $Km^r$ cassette flanked by two *Xba*I sites (Figs. 1A, 2A). In wild-type DNA the *psaF* gene lies on a 1.2 kb *Nco*I restriction fragment, the second *Nco*I site being located 145 bp upstream of the *Cla*I site indicated in Fig. 1B. This fragment is replaced by two *Nco*I fragments of 1.7 kb and 0.9 kb in strain

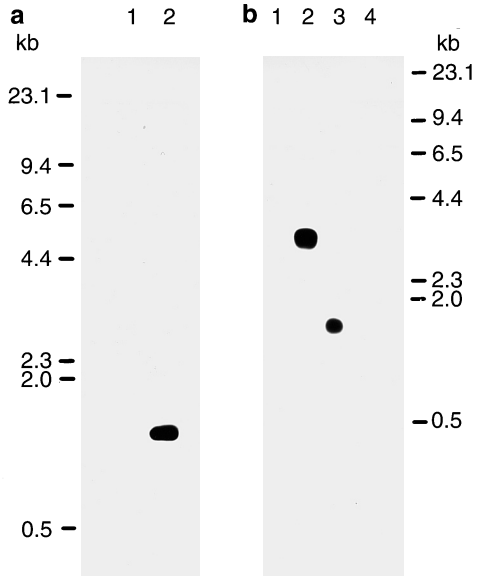


**Fig. 2a, b** Southern blot analysis of DNA from wild-type (wt) and transformant strains of *Syn. elongatus* probed with antibiotic resistance genes used to inactivate PSI subunit-encoding genes. **a** Lane 1, wt DNA × *Xba*I; lane 2, DNA of strain KK2 × *Xba*I; the blot was probed with the *Eco*RI fragment of pRL161 carrying the $Km^r$ gene (Elhai and Wolk 1989). **b** Lane 1, wt DNA × *Eco*RI; lane 2, DNA of strain LS1 × *Eco*RI; lane 3, DNA of strain LC1 × *Eco*RI; lane 4, wt DNA × *Eco*RI, Lanes 1 and 2 were probed with the *Sma*I fragment of pHP45Ω carrying the $Sm^r/Sp^r$ genes (Prentki Krisch 1984); lanes 3 and 4 were probed with the *Hinc*II fragment of pBR325 carrying the $Cm^r$ gene. The restricted DNA was electrophoresed on 0.8% agarose gels, blotted and hybridised as described in Materials and methods. The *Hin*dIII restriction fragments of λ DNA were used as size standards

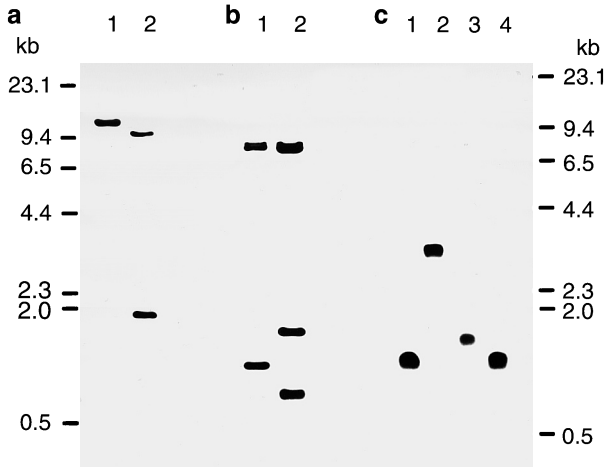


**Fig. 3a–c** Southern blot analysis of the *psaK*, *psaF*, and *psaL* gene loci in wild-type and mutant strains of *Syn. elongatus*. **a** Lane 1, wt DNA × *Xba*I; lane 2, DNA of strain KK2 × *Xba*I; the blot was probed with the *Dde*I fragment carrying *psaK*. **b** Lane 1, wt DNA × *Nco*I; lane 2, DNA of strain FK1 × *Nco*I; the blot was probed with the *Cla*I-*Xho*I fragment carrying the genes *psaF* and *psaJ*. **c** Lane 1, wt DNA × *Eco*RI; lane 2, DNA of strain LS1 × *Eco*RI; lane 3, DNA of strain LC1 × *Eco*RI; lane 4, wt DNA × *Eco*RI; the blot was probed with the *Eco*RI fragment carrying the genes *psaL* and *psaI* genes from *Syn. elongatus*. The mobilities of the *Hin*dIII restriction fragments of λ DNA are indicated on the left **a** and **b** and on the right for **c**

FK1, due to the presence of an additional *Nco*I site in the $Km^r$ gene interrupting *psaF*. As expected from Fig. 1B, both fragments were revealed when the blots were probed with the restriction fragment of pRL 161 used for interrupting the *psaF* gene (not shown). In strain LC1 the 1.3-kb *Eco*RI fragment harbouring the wild-type psaL gene is replaced by two comigrating 1.6-kb *Eco*RI fragments due to the presence of an *Eco*RI site in the $Cm^r$ cassette which replaces an internal segment of *psaL* (Fig. 1C). These fragments also hybridize with a probe for the $Cm^r$ gene of pBR325 (Fig. 2C). Finally, in strain LS1 the 1.3-kb *Eco*RI fragment of wild-type DNA is replaced by a larger fragment (2.9 kb) harbouring the entire $Sm/Sp^r$ cassette and the DNA flanking the *psaL* gene (Figs 2C, 3C). These results, together with other hybridization data (not shown), demonstrate that integrative transformation in *Syn. elongatus* occurs through allele replacement mediated by homologous recombination, as previously observed in mesophilic cyanobacteria (Thiel 1994). In addition, no hybridization signals corresponding to wild-type DNA were detected during the genetic analysis of the transformants, which therefore contained only mutant copies in the polyploid cyanobacterial genome. This indicates that the inactivated PSI subunit genes are not essential for photoautotrophic growth in *Syn. elongatus*, as previously observed for the *psaF* and *psaL* genes in mesophilic cyanobacteria (Chitnis et al. 1993a; Xu et al. 1994). In fact, growth rates corresponding to wild-type levels are observed for all mutants when grown under full illumination (not shown).

The above results were confirmed by the absence of the corresponding subunits from the PSI complexes of the transformant strains as shown in Fig. 4. In the reaction centres of the strains KK1 and FK1 the corresponding subunits PsaK and PsaF are absent, while PSI from strain LS1 lacks the subunit migrating immediately below PsaF. This subunit is N-terminally modified in *Syn. elongatus* and has so far not been unambigously identified (Mühlenhoff et al. 1993). Its absence in the $psaL^-$ strain LS1 indirectly identifies this protein as the *psaL* gene product. In addition, in PSI from the $psaF^-$ mutant FK1 the low molecular weight subunit PsaJ is missing (Fig. 4B). In *Syn. elongatus*, the *psaJ* gene, which is found immediately downstream of *psaF*, is cotranscribed with *psaF* (Mühlenhoff et al. 1993). Thus, the absence of the small PsaJ subunit in PSI complexes from the $psaF^-$ mutant FK1 probably results from transcription termination promoted by the antibiotic marker gene inserted into the proximal *psaF* gene. Indeed this has been observed in a $psaF^-$ mutant of *Synechocystis* sp. PCC 6803 (Xu et al. 1994). For similar reasons the polypeptide seen as

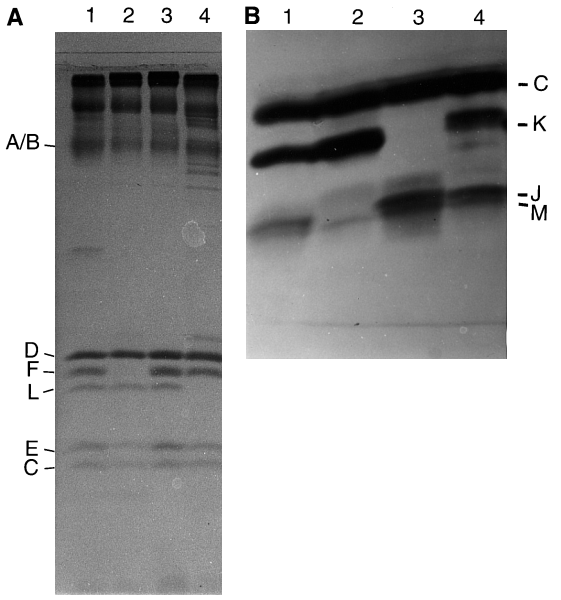


**Fig. 4A,B** SDS-polyacrylamide gel electrophoresis of photosystem I complexes prepared from *Syn. elongatus* wild-type and mutant strains. **A** Gel stained with Coomassie brilliant blue. Samples equivalent to 15 μg chlorophyll were applied per lane. **B** The low molecular weight region of the gel showing subunits of photosystem I visualised by silver staining. Samples equivalent 5 μg of chlorophyll were applied. Lanes 1, wild-type PS I; lanes 2, PS I of strain FK1; lanes 3, PS I of strain KK2; lanes 4, PS I of strain LS1

a faint band above PsaJ, which is apparently missing in the *psaL*$^-$ mutant LS1 might be PsaI, since the corresponding gene is cotranscribed together with *psaL* (Mühlenhoff et al. 1993). However, since this polypeptide is not invariably present in all PSI preparations from wild-type *Syn. elongatus* it cannot unambiguously be assigned to the *psa*I gene.

Molecular masses of approximately 980 kDa were determined by gel filtration experiments for PSI preparations from wild-type *Syn. elongatus* and from the *psaK*$^-$ and *psaF*$^-$ mutant strains, indicating that these preparations contain trimeric PSI (not shown). The fact that the molecular mass for PSI trimers exceeded the previously reported value (Rögner et al. 1990) most probably results from the different gel filtration media utilized in the two studies. For PSI from the *psaL*$^-$ mutant LS1 a molecular mass of approximately 400 kDa was determined, which indicates that this reaction centre was probably extracted as a monomer (not shown). Similar monomeric structures of PSI complexes have been reported previously for a *psaL*$^-$ strain of the mesophilic cyanobacterium *Synechocystis* sp. PCC 6803 (Chitnis and Chitnis 1993). Furthermore, in accordance with earlier observations on PSI monomers obtained by chemical treatments (Rögner et al. 1990), PSI from the *psaL*$^-$ mutant does not bind as strongly to ion-exchange media as PSI trimers. This behavior renders PSI preparations from *psaL*$^-$ mutants difficult to purify to homogenity, since they tend to co-elute with residual PSII from ion-exchange columns. However, our analysis of PSI monomers from *Syn. elongatus* obtained upon deletion of *psaL* did not reveal any variablility in subunit composition of PSI complexes, such as that observed upon PsaL deletion in vitro (Jekow et al. 1995); this suggests that the latter observation probably has no biological significance.

## Transfer and replication of RSF1010-derived plasmids in *Syn. elongatus*

Replicating plasmid vectors, which are essential tools in the highly sophisticated genetics of yeast and *E. coli* and which are increasingly used as extrachromosomal elements in mesophilic cyanobacteria (Thiel 1994), were sought for *Syn. elongatus* as well. However, no endogenous plasmids that could be used for the construction of binary shuttle vectors were detected in this organism, although we cannot completely exclude the possibility that low-copy-number plasmids or easily degraded high molecular weight species might have been missed. Therefore, the replicative properties of promiscuous IncQ plasmids, which are stably maintained in several mesophilic cyanobacteria (reviewed in Thiel 1994), were investigated in *Syn. elongatus*. The RSF1010-derived expression vectors pFC1 harbouring the $\lambda$ phage rightward promotor (Mermet-Bouvier and Chauvat 1994), and pSB2T carrying the *E. coli tac* promotor (Marraccini et al. 1993) were initially introduced into *Syn. elongatus* using electroporation as described above. Cells transformed with the Cm$^r$, Sm$^r$, Sp$^r$ vector pFC1 were selected on Sm medium and were invariably Cm$^r$ and Sp$^r$ also, indicating that they had indeed inherited pFC1 DNA. Plasmid DNA was isolated from several of these Cm$^r$, Sm$^r$, Sp$^r$ clones and independently used to transform *E. coli* to Cm$^r$, Sm$^r$, Sp$^r$ phenotypes. In addition, pFC1 DNA could be detected directly by Southern blot analysis of plasmid DNA preparations from transformed *Syn. elongatus*. As shown in Fig. 5A, a single band corresponding to linear pFC1 DNA was observed upon restiction with *Eco*RI, which cuts pFC1 only once (Mermet-Bouvier and Chauvat 1994). In addition, identical restriction patterns were found for pFC1 replicating in *E. coli* before and after passage through *Syn. elongatus* (see Fig. 5B for typical results). Similar results were obtained with the Cm$^r$, Km$^r$, Sm$^r$, Sp$^r$, plasmid pSB2T (not shown), although selection for Km$^r$ alone proved insufficient for maintenace of pSB2T, which was invariably lost from *Syn. elongatus* upon prolonged cultivation in Km medium. Collectively, these results demonstrate that IncQ plasmids are replicated in *Syn. elongatus* and are stably maintained as long as strong selection pressure is applied.

The vector pSB2T was also used to test the simple biparental mating procedure for conjugative transfer of

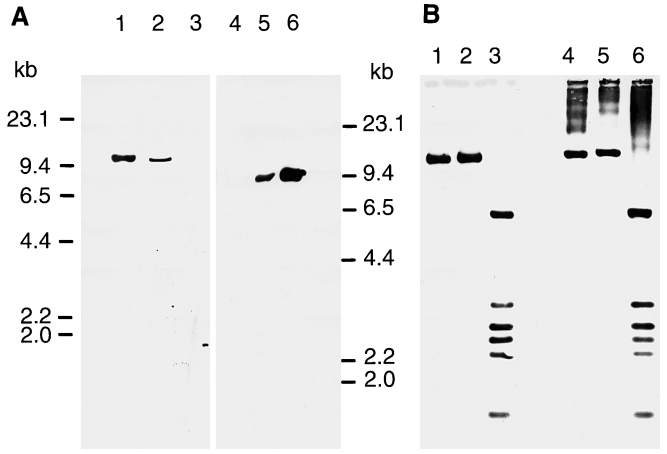


**Fig. 5A, B** Southern blot analysis of the plasmids pFC1 and pSB2T propagated in *Syn. elongatus* and *E. coli*. **A** *Eco*RI digests of pFC1 (lanes 1–3) and pSB2T (lanes 4–6) isolated from *E. coli* strain HB101 and *Syn. elongatus*. Lane 1, pFC1 from *E. coli*; lane 2, plasmid preparation from *Syn. elongatus* strain P1, isolated following an electroporation experiment in the presence of pFC1; lane 3, plasmid preparation from *Syn. elongatus* wild type; lane 4, plasmid preparation from *Syn. elongatus* wild type; lane 5, plasmid-preparation from *Syn. elongatus* strain T0, isolated following a conjugation experiment with *E. coli* CM404 harbouring pSB2T; lane 6, pSB2T from *E. coli*; lanes 1–3 were probed with pFC1 DNA, lanes 4–6 were probed with pSB2T DNA, both isolated from *E. coli* HB101. **B** Restriction analysis of pFC1 isolated from *E. coli* before (lanes 1–3) and after propagation in *Syn. elongatus* (lanes 4–6). The following restriction enzymes were used: lanes 1 and 4: *Eco*RV; lanes 2 and 5, *Sma*I; lanes 3 and 6, *Hin*fI. Approximately 1 μg of plasmid DNA was used for each digest, except for *Hin*fI where 2 μg DNA was used. The restriction fragments were separated on an 1.2% agarose gel, blotted and probed with biotinylated pFC1 DNA isolated from *E. coli*

RSF1010-derivatives from *E. coli* to *Syn. elongatus* (see Materials and methods). Again, initial Km selection was inefficient and cells with $Cm^r$, $Sm^r$, $Sp^r$ phenotypes were obtained by Sm selection. pSB2T DNA could be directly detected in plasmid preparations from these cyanobacterial clones by Southern blot analysis (Fig. 5A) and could be successfully reintroduced into *E. coli*. However, we presently see no real advantage over electroporation in the use of conjugation for the transfer of RSF1010-derived plasmids to *Syn. elongatus*. The copy number of these RSF1010 derivatives in the thermophilic strain was not determined. A value of about 10 copies per cell as previously determined for several mesophilic cyanobacteria, seems a reasonable estimate since the amount of plasmid DNA isolated routinely from small-scale cultures was invariably too low to allow visualisation by ethidium bromide staining (Mermet-Bouvier et al. 1993). Finally, pFC1 DNA prepared from *Syn. elongatus* was used to confirm that the host defence systems of *E. coli* do not strongly interfere with the establishment of plasmids isolated from this cyanobacterial host, with the possible exception of EcoK, which is deleted in most *E. coli* laboratory strains and thus was not tested. The DNA preparations were found to transform the various *E. coli* laboratory strains tested equally well (not shown); XL1 blue ($EcoK^-$, $mcrA^+$, $mcrBC^+$, $mrr^+$), ER 1565 ($EcoK^-$, $mcrA^-$, $mcrBC^-$, $mrr^+$) and ER1647 ($EcoK^-$, $mcrA^-$, $mcrBC^-$, $mrr^-$). These results are not unexpected in view of the fact that genomic DNA libraries from *Syn. elongatus* could be efficiently introduced and propagated in *E. coli*. We could not investigate the possible existence of *Syn. elongatus* restriction barriers for DNA replicated in *E. coli* since the cyanobacterial transformants cannot be selected directly on solid medium, thus rendering the quantification of transformation efficiencies difficult. Restriction barriers should, however, be taken into consideration when cloning into this bacterium from which at least one restriction enzyme has been characterized (Miyake et al. 1992).

## Discussion

We have shown that DNA can be introduced into the thermophilic cyanobacterium *Syn. elongatus* by electroporation or conjugation and that the genome of this organism can be manipulated through integrative transformation. Upon targeted inactivation of the *psaL* and *psaF* genes we were able to confirm the results previously obtained for similar mutations in PSI from mesophilic cyanobacteria, indicating that *Syn. elongatus* is a suitable model organism for site-directed mutagenesis of photosystem I, and we report the first characterization of a $PsaK^-$ PSI complex. In addition, we have shown, using two broad host-range expression vectors for mesophilic cyanobacteria that RSF1010-derived plasmids can be shuttled unaltered between *Syn. elongatus* and *E. coli*. Therefore, these plasmids can be used for in vivo studies of cyanobacterial proteins in both mesophilic and thermophilic backgrounds Marraccini et al. 1993; Mermet-Bouvier and Chauvat 1994).

The establishment of gene manipulation techniques in *Syn. elongatus* was no trivial undertaking. For instance, it was not certain whether the antibiotics used in this study would be stable enough at elevated temperatures to ensure effective selection of transformants. This phenomenon of instability may explain the loss of plasmids observed under kanamycin selection. Similarly, it could not be taken for granted that the antibiotic marker genes originating from mesophilic bacteria would be expressed at high levels enough to confer antibiotic resistance on *Syn. elongatus* cells, nor that the encoded enzymes would be sufficiently active at the required elevated growth temperatures. Finally, the possible existence of restriction barriers to foreign DNA propagated in *E. coli* has never been investigated for this thermophilic strain. In view of these possible pitfalls it is very encouraging that *Syn. elongatus* can now be genetically manipulated by standard techniques. However, antibiotic resistance selection has to be improved for successful application to cells growing

on solid media, so as to determine the efficiencies of transformation. We are currently investigating the physiology of this organism, of which very little is known and which even remained refractory to plating for a long time (Castenholz 1981). In this context we observed that under our growth conditions *Syn. elongatus* grows as rather fragile filaments, similar to *Phormidium laminosum* (Rippka et al. 1981). When cultures are carefully supplemented with air, short filaments of 6 to 10 cells constitute the major part of the population. Apparently, this strain, which has so far been classified as unicellular, is of filamentous heritage (Yamaoka et al. 1978). In addition, nucleotide sequences of homologous genes from *Syn. elongatus* and *Syn. vulcanus* display 100% identity in coding and adjacent noncoding regions (Mühlenhoff et al. 1993; Shimizu et al. 1990, 1992; Golbeck 1994). This strongly suggests that these two thermophilic strains, which were isolated from distant parts of the world, correspond to a single organism. Accordingly, we will make every effort to cooperate with other workers in resolving the confusion surrounding the utilisation of the names *Synechococcus elongatus* (Miyake et al. 1992) and *Synechococcus vulcanus* (Koike et al. 1989).

In conclusion, *Syn. elongatus* is, to the best of our knowledge, the first thermophilic phototroph which has been successfully manipulated by genetic means. We hope that this work will make an important contribution towards future investigations of structure-function relationships of plant-like oxygenic photosynthesis in this organism, which provided the most detailed crystal structure of photosystem I currently available (Krauss et al. 1993). In addition, the present report should encourage the development of genetic systems for other thermophilic cyanobacteria as well as for phototrophic organisms belonging to other bacterial genera with predominantly thermophilic isolates, such as the green non-sulfur bacteria (Kondratieva et al. 1989).

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