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Highly efficient integration of foreign DNA into the genome of the green sulfur bacterium, *Chlorobium vibrioforme* by homologous recombination

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Abstract

Highly efficient and reproducible transformation of *Chlorobium vibrioforme* with plasmid DNA has been achieved by electroporation. Specific parameters have been optimized for the electrotransformation procedure. The method was developed using a construct containing a full copy of the *pscC* gene encoding the cytochrome c_{551} subunit of the photosynthetic reaction center complex and the *aadA* gene encoding streptomycin resistance as selectable marker. Southern blotting analysis showed that the tested colonies were true transformants with the plasmid integrated into the genome by single homologous recombination. No transformants were obtained using the vector without the *pscC* gene showing that this vector does not replicate in *C. vibrioforme*. Thus transformation is possible only by homologous recombination. When using constructs designed to inactivate the *pscC* gene by insertion no transformants were obtained, indicating that the gene is indispensable for growth. The vector pVS2 carrying genes for erythromycin and chloramphenicol resistance was shown to replicate in *C. vibrioforme*. The two transformations shown here, provide an important genetical tool in the further analysis of structure and function of the photosynthetic apparatus in green sulfur bacteria.

Abbreviation: Bp – base pairs

Introduction

Photosynthetic reaction centers of different purity have been prepared from *Chlorobium* species (Feiler et al. 1992; Miller et al. 1992; Okkels et al. 1992; Oh-oka et al. 1993). Their characterization has identified these green sulfur bacteria as useful model systems for Photosystem I in higher plants. The reaction center complex contains electron acceptors with similarities to the iron sulfur centers F_A, F_B and F_X in Photosystem I, as evidenced by electron paramagnetic resonance spectroscopy (Nitschke et al. 1990; Feiler et al. 1992; Miller et al. 1992; Oh-oka et al. 1993; Kjær et al. 1994). Similarities of the *Chlorobium* reaction center complex to Photosystem I are apparent at the polypeptide sequence level (Büttner et al. 1992). In spite of the structural similarity to PS I, the photosynthetic reaction center complex of *Chlorobium* contains unique elements such as a membrane-bound cytochrome c_{551}. The gene, now designated *pscC* (Bryant 1994), encoding this cytochrome c_{551} has been characterized and found to encode a unique cytochrome with a deduced molecular mass of 23 kDa and with a single heme group serving as the electron donor to the photooxidized primary donor, P840 (Okkels et al. 1992). Not only the reaction center complex but also the light harvesting antenna system of green sulfur bacteria has received a recent focus of attention. In particular, the organization of pigments in the chlorosomes is a matter of debate (Wullink et al. 1991) and the energy transfer to

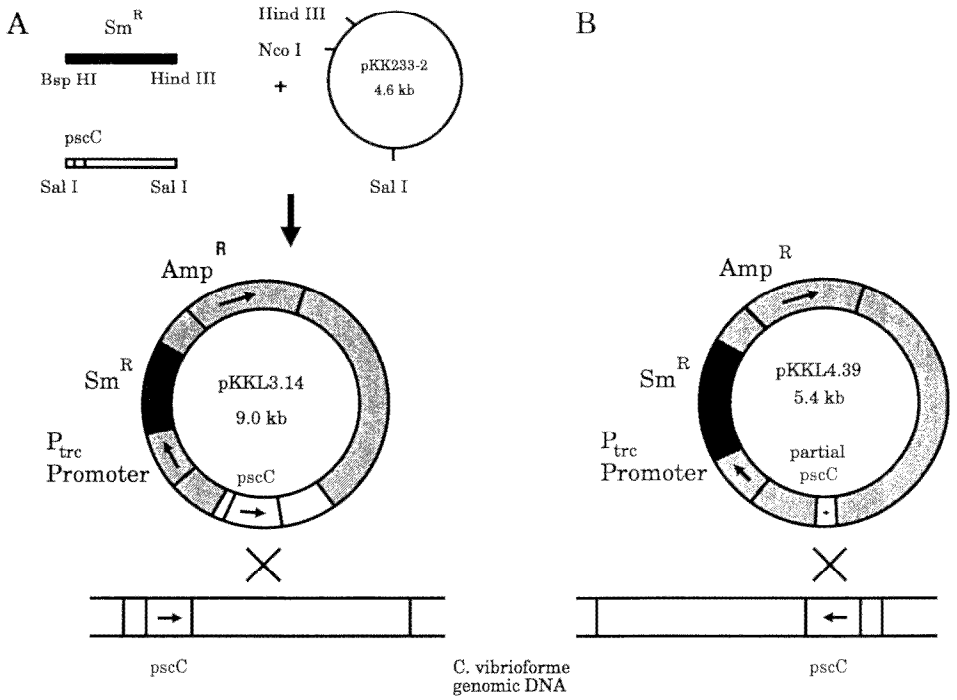

Fig. 1. Constructs used for transformation of *C. vibrioforme*. Panel A: The plasmid pKKsm was digested with *SalI* and ligated with a 3.6 kb *SalI* fragment containing the *pscC* gene encoding the cytochrome c_{551} from *C. vibrioforme* (Okkels et al. 1992). Panel B: The plasmid pKKL4.39 was constructed to inactivate the *pscC* gene. The white box corresponds to a 400 bp fragment of the *pscC* gene encoding the N-terminal half of the cytochrome c_{551}. No transformants were obtained using this construct.

the reaction center has turned out to be redox regulated by an as yet unknown mechanism (Blankenship et al. 1993).

The characterization of the photosynthetic apparatus of *Chlorobium* and of some of its polypetide components calls for a method to transfer modified genes into these organisms. *Chlorobium vibrioforme* permits the transfer of homologous chromosomal DNA into the cell by natural transformation and appears competent throughout its entire cell cycle (Ormerod 1988). A draw-back of the original method is the low and inconsistent transformation efficiency.

In this paper, we report a highly efficient and reproducible transformation method based on electroporation. DNA integration is subsequently achieved by homologous recombination into the genome of *C. vibrioforme*. Transgenic cells were obtained using a construct containing the *pscC* gene and the transposon Tn7 *aadA* gene encoding for streptomycin adenylyltransferase (Fling et al. 1985) as selectable marker. We also demonstrate plasmid transformation with the pVS2 vector which does not carry *C. vibrioforme* DNA.

Materials and methods

Bacterial strains and growth

Chlorobium vibrioforme strain NCIB 8327 was grown in screw capped bottles in the TA medium of Sirevåg and Ormerod (1970) as described by Rieble et al. (1989) at 28 °C in light provided by incandescent bulbs (30–50 μmol photons $m^{-2}s^{-1}$, 400–700 nm). The organism was plated on TA-agar plates (Ormerod 1988) in an Mark 3 anaerobic work station (Don Whitley Scientific Ltd., West Yorkshire, UK). All manipulations of cells during transformation were carried out under anaerobic conditions.

Escherichia coli strains XL1-blue (Stratagene, CA) and HB101 (Bolivar and Backman 1979) were used for all recombinant DNA manipulations.

Construction of the plasmid pKKL3.14

The expression plasmid pKK233–2 (Pharmacia) was digested with *Nco*I and *Hin*dIII and the fragment removed was replaced with a 0.8 kb *BspHI-Hin*dIII PCR amplified fragment of the bacterial transposon

Tn7 *aadA* gene encoding for streptomycin and spectinomycin resistance ($sm^r/spec^r$) (Fling et al. 1985). This inserted the *aadA* gene directly behind the constitutive P_{trc}-promoter. The construct, designated pKKsm, was digested with *SalI* and ligated with a 3.6 kb *SalI* fragment containing the *pscC* gene from *C. vibrioforme* (Okkels et al. 1992). The obtained plasmid pKKL3.14 (Fig. 1) was used for the transformation experiments. It was propagated in *E. coli* (XL1-blue) grown in LB medium supplemented with ampicillin (50 $\mu g ml^{-1}$). Standard molecular biological methods were performed according to Sambrook et al. (1989).

Natural transformation

A 100 μl aliqout of an exponential phase culture of *C. vibrioforme* (15 h old) grown in light at 28 °C was mixed with 30 ng DNA of plasmid pKKL3.14 in 10 ml TA medium. After 15 h incubation in light at 28 °C, 50 μl of the culture were plated out on TA-agar plates containing 60 $\mu g\ ml^{-1}$ streptomycin and 60 $\mu g\ ml^{-1}$ spectinomycin. The plates were incubated for 7 days in light at 28 °C.

Plasmid pVS2 (Von Wright et al. 1987) was obtained from Dr T. W. Aukrust, Norwegian Food Research Institute, N-1430 Aas, Norway. The plasmid was amplified in *E. coli* (HB101) in LB medium supplemented with chloramphenicol (15 $\mu g\ ml^{-1}$) and the purified plasmid was used to transform *C. vibrioforme* on plates of nonselective agar (Ormerod 1988). After incubation for 24 h, the cells were transferred to agar plates containing chloramphenicol (10 $\mu g\ ml^{-1}$) or erythromycin (5 $\mu g\ ml^{-1}$). Putatively transformed colonies were tested for antibiotic resistance and for the presence of pVS2 by inoculating TA medium with antibiotic with a colony and performing a plasmid purification of the grown culture.

Electroporation

C. vibrioforme cells in the stationary phase (3 ml) were used to inoculate 150 ml TA medium in a screw capped bottle and the culture was illuminated at 28 °C for 15 h. The cells were harvested by chilling the culture on ice for 0.5–1 h and centrifuging at 12 000 *g* for 12 min at 4 °C. The pelleted cells were washed twice with ice cold deionized H_2O and finally resuspended with 2 ml ice-cold deionized H_2O or 2 ml ice-cold 270 mM sucrose to give a final concentration of $\sim 10^9$ cells/ml. The cell aliquot (100 μl) to be transformed was mixed with 30–50 ng plasmid pKKL3.14 in a 0.2 cm electrode gap cuvette. The electroporation was carried out by discharging a single pulse. The Gene Pulser apparatus (Bio-Rad Laboratories) was set at 25 μF, while the voltage and the value of the parallel resistor on the Pulse Controller (Bio Rad Laboratories) were varied. Immediately after discharging, the sample was transferred to 10 ml TA medium. After incubation for 15 h at 28 °C in light, the cells were plated out on selective medium as described for natural transformation, and incubated for an additional 7 days at 28 °C in the light.

Southern blotting

Total genomic *C. vibrioforme* DNA was isolated according to Wilson (1993). The DNA was separated by electrophoresis in 0.3 and 0.9% agarose gels. The blotting was carried out using 0.4 M NaOH as transfer buffer. Hybridizations were carried out at 68°C in 10% dextran sulfate, 1.5 × SSPE (0.27 M NaCl, 15mM Na_2HPO_4, and 1.5 mM EDTA, pH 7.0), 1% SDS and 0.5% skimmed milk powder. A 1.5 kb fragment of the *aadA* gene and a 650 bp fragment of the *pscC* gene were labeled with [α-^{32}P]dCTP and used as probes.

Results

The natural transformation system was used to transform the plasmid pKKL3.14 into the genome of *C. vibrioforme*. However, the efficiency was low and variable. This prompted us to investigate electroporation as a potential method to introduce DNA into cells. The high-field pulse given by the electroporation method is believed to produce transient pores at protein-lipid junctions in the cell membrane and thus to allow uptake of exogenous DNA. Most prokaryotic cells are electrotransformed with high efficiency at a field strength of 12.5 kV cm^{-1} and a capacitor setting of 25 μF (Dower et al. 1988). When these same parameters were used for electrotransformation of *C. vibrioforme*, only a few transformants were obtained. Consequently, to determine the optimal electroporation parameters for *C. vibrioforme*, a series of experiments using different field strengths and time constants was carried out. A field strength of 7500 V cm^{-1} with a pulse duration of 15 msec resulted in maximum transformation efficiency (Fig. 2).

The colonies obtained after growth on sm/spec plates were analyzed by Southern blotting. Total DNA from the wild type and transformants was digested with *Hin*dIII and probed with a 650 bp *SalI-Pvu*II frag-

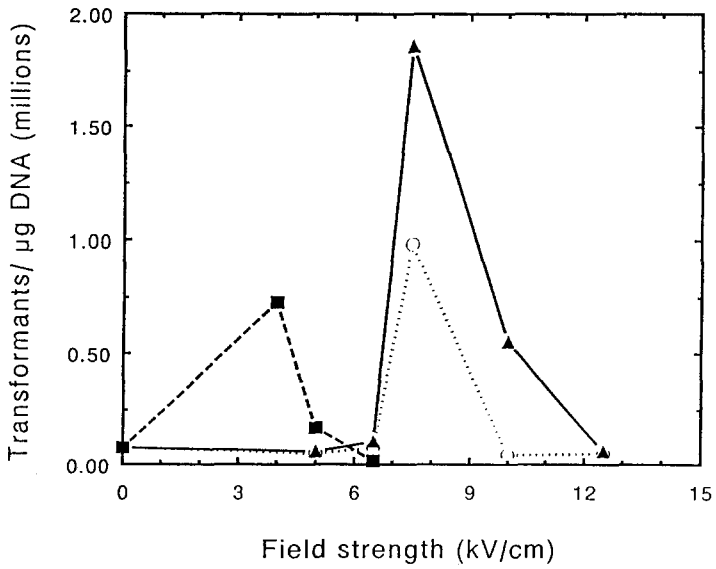

Fig. 2. Effect of field-strength and pulse duration on electro-transformation of *C. vibrioforme*. The electroporation was carried out by discharging a single pulse in a 0.2 cm electrode gap cuvette using pulses of 5 msec (○), 15 msec (▲) or 54 msec (■).

ment of the *pscC* gene encoding for the N-terminal half of the cytochrome c_{551} and a 1.5 kb *Ssp*I fragment of the *aadA* gene. As expected, the *pscC* probe fragment hybridizes to a 0.6 kb band and a 1.1 kb band in the wild type (Fig. 3A). Two different transformant strains were tested and the predicted additional hybridization band of 1.6 kb was detected (Fig. 3A). The expected hybridization bands of 4.3 kb and 1.6 kb were detected using the 1.5 kb *Ssp*I fragment of the *aadA* gene as probe in the two independent transformants (Fig. 3B). No hybridization bands were detected in the wild type using the 1.5 kb *aadA* fragment as probe. Undigested total genomic DNA of transformants showed a hybridization band migrating with the genomic DNA different from the undigested plasmid pKKL3.14 hybridization bands (Fig. 3C).

The Southern blots in Fig. 3 show that the electro-transformed plasmid pKKL3.14 has been integrated into the genome of *C. vibrioforme* by homologous recombination. No transformants were obtained on selective plates using the pKKsm plasmid which carries resistance genes but no *C. vibrioforme* DNA (data not shown). It can therefore be concluded that integration of foreign DNA into the genome of *C. vibrioforme* requires the presence of homologous DNA. This demonstrates that transformation with pKKsm is possible only by homologous recombination. The pKKsm vector is not replicated in *C. vibrioforme*.

The pVS2 streptococcal cloning vector carries genes for erythromycin and chloramphenicol resistance. After transformation and 7 days incubation on selective medium, a few transformants appeared on the erythromycin plates but none on the chloramphenicol plates. Two of the colonies from the erythromycin plates were examined further and found to be resistant to erythromycin (20μg ml^{-1}) whereas their resistance to chloramphenicol was only slightly increased compared to the wild type. Both transformants contained plasmid DNA corresponding to pVS2 (data not shown).

Discussion

Efficient transformation of *C. vibrioforme* has been achieved by electroporation after careful optimization of field strength and pulse duration (Fig. 2). Standard electroporation conditions for *E. coli* resulted in only few transformants and the electroporation parameters must be controlled within a narrow range. With the conditions described here, the transformation efficiency is reproducible and gives a high number of transformants.

The integration of the recombinant plasmid pKKL3.14 into the chromosomal copy of the *pscC* gene was achieved by a single crossover event. After integration, a full copy of the *pscC* gene is retained (Fig. 3A). Different constructs predicted to cause inser-

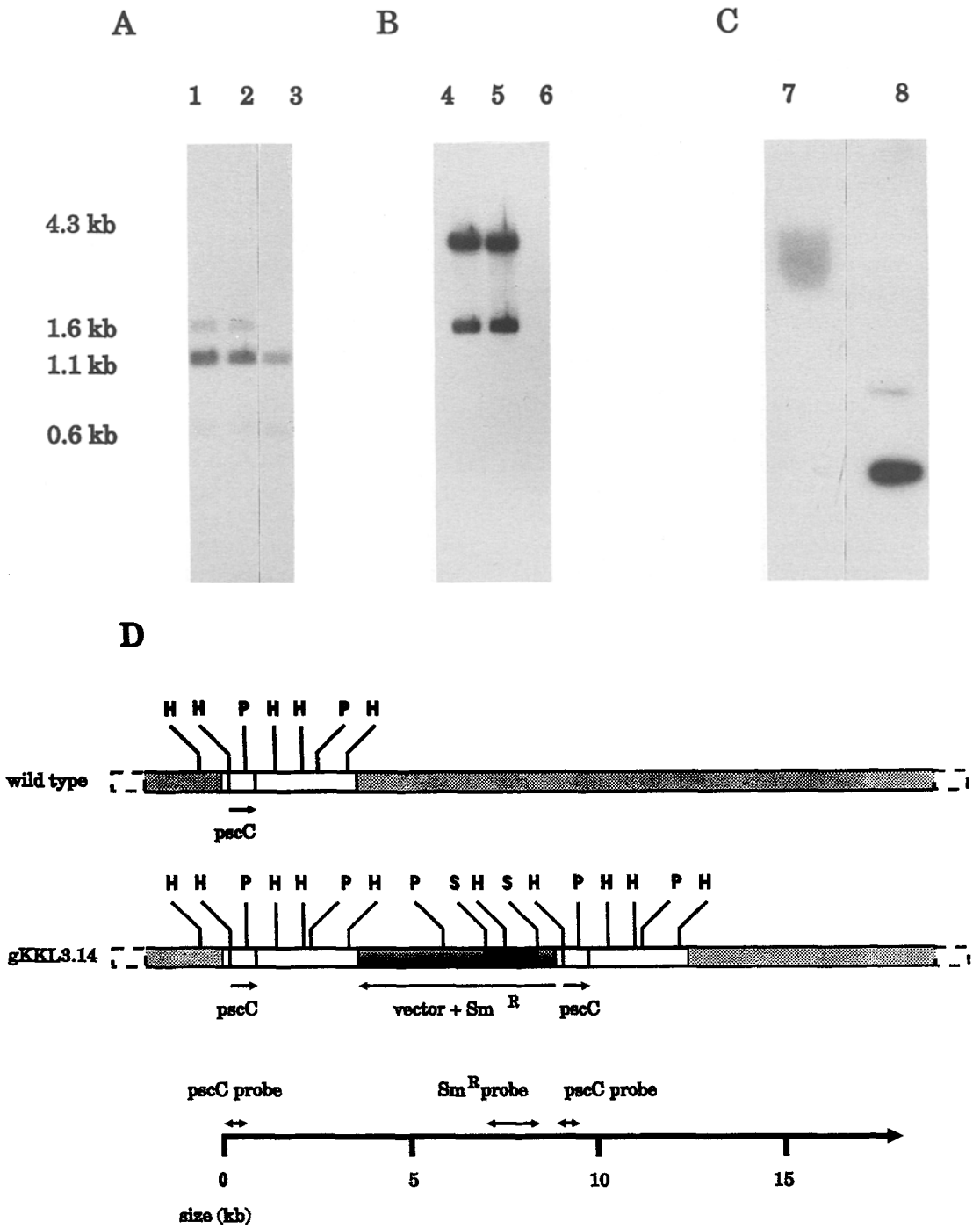

Fig. 3. Southern blot analysis of total DNA isolated from *C. vibrioforme* wild type and from transformants obtained on selective plates after electroporation with the pKKL3.14 plasmid. Panel A: Lane 1 and 2 contained DNA from two independent transformants digested with *Hin*dIII. Lane 3 contained DNA from the wild type digested with *Hin*dIII. All lanes contained the same amount of DNA and were electrophoresed in a 0.9% agarose gel. A 650 bp *Sal*I-*Pvu*II fragment of the *pscC* gene encoding for the N-terminal half of the cytochrome c_{551} was used as probe. Panel B: Lanes 4, 5 and 6 represents the same filter used in panel A, lanes 1, 2 and 3. The filter was stripped off and reprobed with the 1.5 kb fragment of the *aadA* gene. Panel C: undigested total DNA from resistant colonies (lane 7) and the undigested pKKL3.14 plasmid (lane 8, detected as supercoiled and nicked circle) electrophoresed in a 0.3% agarose gel. A 1.5 kb fragment of the *aadA* gene was used as probe. Panel D: Relevant restriction enzyme sites in the wild type and in the transformants. The white box corresponds to the *Sal*I fragment of *C. vibrioforme* DNA inserted in the vector. Restriction endonucleases: H, *Hin*dIII; P, *Pvu*II; S, *Ssp*I.

tional inactivation in the *pscC* gene were also made. Using such constructs e. g. the pKKL4.39 plasmid shown in Fig. 1B, no recombinant cells were obtained. This suggests that the *pscC* gene is indispensable in *C. vibrioforme*. This is not surprising considering the function of cytochrome c_{551} as the primary electron donor to the photosynthetic reaction center (Okkels et al. 1992) and suggests that *C. vibrioforme* does not contain an alternative electron donor to the reaction center. This is in contrast to the situation in purple bacteria such as *Rhodobacter capsulatus* which contains an alternative electron donor cytochrome c_y (Jenney and Daldal 1993).

The results show that *C. vibrioforme* can be transformed with the pVS2 plasmid vector containing an origin of replication from *Streptococcus lactis*, but at low frequency. Most likely the efficiency of transformation with the replicating pVS2 plasmid could also be improved by using electroporation to target the DNA. Resistance to erythromycin, but not to chloramphenicol, is well expressed under the conditions used.

Generally, spontaneous resistance mutants can arise and therefore it is important to control by Southern blotting or PCR analysis whether the resistant phenotype results from the selectable marker gene. The Southern blotting analysis clearly confirms that the *aadA* selectable marker gene is integrated in the genome of *C. vibrioforme* and that electroporation conditions and subsequent selection did not result in spontaneous resistance. The transposon Tn7 *aadA* gene confers resistance to both streptomycin and spectinomycin. When both antibiotics are used in combination, spontaneously resistant mutants are less likely to occur. When using streptomycin and spectinomycin at 20 μg ml^{-1}, colonies without the *aadA* gene were occasionally observed. At the higher concentrations routinely used, spontaneously resistant mutants were never observed.

A single-crossover event is not stable following growth in non-selective medium because the vector is likely to be excised from the genome, thereby causing a reversion to wild type. Therefore it is necessary to grow the transformed cells under continuous selection for resistance. In contrast, a double-crossover event can be designed to create a deletion in a part of the genome, thus preventing the reversion to wild type in non-selective medium. We are currently exploring the function of reaction center polypeptides of *C. vibrioforme* by using constructs leading to double crossover events.

The two transformation methods reported here provide very strong tools to genetically investigate the molecular processes in the photosynthetic light-harvesting antennae and reaction center of *C. vibrioforme*. Mutant strains with deleted genes may be obtained by transformation and homologous recombination using the pKKsm vector. Subsequently site-directed mutations in the genes may be investigated in vivo by transformation using the pVS2 vector.

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