Insertional inactivation studies of the *csmA* and *csmC* genes of the green sulfur bacterium *Chlorobium vibrioforme* 8327: the chlorosome protein CsmA is required for viability but CsmC is dispensable

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Abstract

Targeted mutagenesis was used to investigate the roles of the CsmA and CsmC proteins of the chlorosomes of the green bacteria *Chlorobium tepidum* and *Chlorobium vibrioforme* 8327. Under the photoautotrophic growth conditions employed, CsmA is required for the viability of the cells but CsmC is dispensable. The absence of CsmC caused a small red shift in the near-infrared absorption maximum of bacteriochlorophyll *d* in whole cells and chlorosomes, but chlorosomes were assembled in and could be isolated from the *csmC* mutant. The doubling time of the *csmC* mutant was approximately twice that of the wild-type strain. Fluorescence emission measurements suggested that energy transfer from the bulk bacteriochlorophyll *d* to another pigment, perhaps bacteriochlorophyll *a*, emitting at 800–804 nm, was less efficient in the *csmC* mutant cells than in wild-type cells. These studies establish that transformation and homologous recombination can be employed in targeted mutagenesis of *Chlorobium* sp. and further demonstrate that chlorosome proteins play important roles in the structure and function of these light-harvesting organelles. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Chlorosomes are photosynthetic light-harvesting complexes found only in green bacteria [1,2]. These sac-like organelles, which occur tightly appressed to the inner surface of the cytoplasmic membrane, contain highly aggregated, rod-shaped arrays of bacteriochlorophyll (*Bchl*) *c*, *d*, or *e* dependent upon the source organism, and additionally contain proteins, carotenoids, glycolipids, and quinones as major constituents. Electron microscopy, protease susceptibility mapping, and agglutination experiments using a
variety of antisera suggest that chlorosomes are surrounded by a protein- and lipid-containing, monolayer envelope [3–7]. Highly purified chlorosomes from *Chlorobium tepidum* have been shown to contain 10 different proteins [8–10], and the genes for most of these have been cloned and characterized [6–10]. However, the specific functional roles of these proteins in chlorosome structure, function, and biogenesis remain unknown.

In studies of phycobilisomes, the light-harvesting antenna structures of cyanobacteria, targeted or interposon mutagenesis was a very effective method for the elucidation of the functional roles of the component proteins of these structures (for reviews, see [11,12]). In this report we describe the first application of targeted mutagenesis to the study of chlorosome structure and function and describe the properties of a *csmC* mutant lacking the 14-kDa protein of the chlorosomes. These experiments establish the occurrence of homologous recombination in *Chlorobium* sp. and the feasibility of using targeted mutagenesis to study the roles of proteins in the structure, function, and biogenesis of chlorosomes.

2. Materials and methods

*Chlorobium vibrioforme* strain 8327 and *Chlorobium tepidum* were grown as previously described [13,14]. All DNA manipulations were performed in *Escherichia coli* strain DH5α (Gibco-BRL, Gaithersburg, MD). *E. coli* strains were grown in Luria-Bertani medium [15]; when appropriate, the medium was supplemented with ampicillin (100 μg ml⁻¹), kanamycin (40 μg ml⁻¹) or streptomycin (30 μg ml⁻¹). Plasmids pUC18 or pUC19 were employed for subcloning, interposon mutagenesis, and DNA sequencing. The cloning and characterization of the *csmCA* operons of *Ch. vibrioforme* and *C. tepidum* have been described [8]. DNA fragments encoding resistance to streptomycin/spectinomycin (the Ω cassette [16]) or to kanamycin (the aphII gene that encodes aminoglycoside 3’-phosphotransferase II [17]) were used to perform insertional inactivation of the *csmA* and *csmC* genes. Southern blot hybridization analyses were performed as described [8].

Transformation of *Cb. vibrioforme* was performed as described [13,18]. Cells were mixed with a loop-full of either plasmid or linearized DNA fragment suspension and plated on a non-selective plate as a thick patch and incubated in the light for 1–3 days. After the cells had grown, transformants were selected on agar plates (*Chlorobium* medium containing 1.5% (w/v) Bacto agar including antibiotics. Antibiotic resistant transformants were selected on agar plates containing 15 μg ml⁻¹ streptomycin and 15 μg ml⁻¹ spectinomycin or 40 μg ml⁻¹ kanamycin. The plates were incubated under the light for 1–3 days in an anaerobic controlled environment chamber in an atmosphere of 85% N₂, 10% CO₂, and 5% H₂. Growth curves for liquid cultures were generated by monitoring the optical density of cultures at 650 nm.

Optical density and absorption measurements were performed with a Cary 14 spectrophotometer that was modified for computerized data acquisition by On-Line Instruments Systems (Bogart, GA). Fluorescence emission spectra at 77 K were obtained with an SLM-Aminco 8000C fluorometer with excitation at 449 nm. Protein concentrations were determined by the method of Bradford [19] using the BCA reagents purchased from Pierce Chemical Co. (Rockford, IL). Bchl concentrations were determined by extraction with aqueous acetone (80% v/v) using the specific absorption coefficient 92.6 l (g cm)⁻¹ for Bchl *c* or 98.0 l (g cm)⁻¹ for Bchl *d* [20]. Protein compositions were analyzed by polyacrylamide gel electrophoresis as described [7,21]. Immunoblotting
with antisera prepared against recombinant chlorosome proteins was performed as described [6,7].

3. Results and discussion

To investigate the roles of CsmA and CsmC, the csmA and csmC genes were insertionally inactivated by interposon mutagenesis with the Ω cassette [16], encoding resistance to streptomycin and spectinomycin, or the aphII gene, encoding aminoglycoside 3’-phosphotransferase and conferring resistance to kanamycin [17] (see Figs. 1A and 2A). In plasmids pCB336 and pCB337, the csmA gene of Cb. vibrioforme is insertionally inactivated by the introduction of the Ω cassette in either orientation into the unique NcoI site within the coding sequence of that gene (Fig. 1A). Similarly, plasmids pCB633 and pCB634 contain the Ω cassette inserted in either orientation in an AvaI site within the coding sequence of the csmC gene of Cb. vibrioforme; plasmid pCB626 contains the aphII gene inserted into the same site (Fig. 2A). These plasmids were used to transform Cb. vibrioforme cells, and transformants resistant to streptomycin/spectinomycin or kanamycin as appropriate were selected. Transformants selected for detailed studies were identified by the number of the plasmid number used to create them.

Southern blot hybridization analyses with DNA isolated from Cb. vibrioforme transformants 336 and 337 confirmed that homologous recombination of the appropriate linearized plasmid and the chromosomal csmA locus had occurred (Fig. 1B,C), but that segregation of the csmA and csmA:Ω alleles had not occurred. As shown in Fig. 1A,B, lane 1, the csmA gene is encoded on a 1.57-kb HindIII fragment. Insertion of the Ω cartridge at the unique NcoI site within the csmA coding sequence introduced two additional HindIII sites as indicated in Fig. 1A. For all transformants tested, the hybridization pattern showed the presence of both the 1.57-kb HindIII fragment wild-type fragment as well as the two smaller HindIII fragments of 0.85-kb and 0.72-kb that arise from the introduced HindIII sites that flank the Ω cartridge in the insertionally inactivated csmA gene. Even after repeated restreaking and growth on up to 600 μg ml⁻¹ streptomycin (Fig. 1B,C, lanes 4), no segregation of alleles occurred. To show that the antibiotic resistance of the mutants was not the result of a spontaneous mutation, Southern hybridization experiments were also performed with a probe specific for the Ω cartridge. As shown in Fig. 1C, lanes 2–4, a 2.0-kb HindIII fragment in the DNA isolated from each of the transformants hybridized to this probe, confirming that homologous recombination had occurred and that the antibiotic resistance was due to the Ω cassette. A similar set of experiments was performed with the csmA gene of Cb. tepidum, and identical results were obtained (data not shown). In other organisms the failure of alleles to segregate has been taken to be strong presumptive evidence for the essentiality of the product of the gene in question [22]. Thus, we conclude that, under the photoautotrophic growth conditions employed in these experiments, CsmA is required for viability of Cb. vibrioforme and Cb. tepidum.

The requirement of the csmA gene product for viability was unexpected, since in other photosynthetic bacteria, photosynthesis and photoautotrophic growth can still occur in the complete absence of light-harvesting antenna complexes, as long as sufficient light is provided. For example, a cyanobacterial mutant lacking detectable phycobiliproteins was still capable of photoautotrophic growth [23], and many mutants lacking various light-harvesting proteins in purple bacteria have been described [24]. Foidl et al.
[25] have recently shown that chlorosomes and chlorosome proteins are present even in chemotrophically grown cells of *Chloroflexus aurantiacus*. The ratio of these proteins to the specific Bchl c content of the cells changed approximately 8–10-fold when cells were shifted from aerobic/chemotrophic to...
anaerobic/phototrophic growth conditions. These studies show that the synthesis of chlorosome poly-peptides is largely independent of Bchl c synthesis. In \textit{Cb. vibrioforme} anaesthetic gases, such as \textit{N}_2\textit{O}, acetylene, and ethylene, can be used to inhibit the synthesis of Bchl \textit{d} and chlorosomes [26], yet the chlorosome proteins are still present in such cells in large amounts (V. Nguyen and J.G. Ormerod, unpublished results). These observations are consistent with the idea that chlorosomes and/or specific chlorosome proteins may have an important cellular function(s) other than light-energy harvesting. Since reaction center preparations of green sulfur bacteria typically contain substantial amounts of the FMO protein, which forms the baseplate of the chlorosome [2,27], one intriguing possibility is that components of the chlorosome envelope, including CsmA, are required for the proper structural organization of the proteins on the acceptor side of the reaction center complex. Alternatively, biogenesis of the photosynthetic apparatus in \textit{Chlorobium} species may be dependent upon the assembly of the chlorosome envelope. However, further experiments on the localization and functional role(s) of CsmA will be required to elucidate its function.

In contrast to the results obtained with attempts to inactivate the \textit{csmA} genes, Southern blot hybridization experiments with DNA isolated from \textit{Cb. vibrioforme} transformants 633 and 634 showed that the \textit{csmC} and \textit{csmC}::\Omega alleles had segregated completely (Fig. 2B,C). As shown in Fig. 2B, when DNA the transformant strains was digested with \textit{NcoI} and \textit{SalI}, no evidence for the presence of the 1.45-kb wild-type fragment (Fig. 2B, lane 1) was observed in these strains (compare Fig. 2B, lanes 1–3). Similarly, \textit{Cb. vibrioforme} transformant 626, in which the \textit{csmC} gene was insertionally inactivated with the \textit{aphII} gene, also segregated completely (compare Fig. 2B, lanes 4 and 5). For each transformant, hybridization experiments performed with DNA fragments encoding the appropriate drug resistance marker confirmed that the antibiotic resistance was due to the presence of the appropriate DNA cassette (Fig. 2C, lanes 2, 3 and 5). These experiments again confirm that homologous recombination occurs in \textit{Cb. vibrioforme} and that CsmC is not essential under the photoautotrophic growth conditions employed during the isolation of these mutant strains. These results additionally show that neither the \textit{aphII} nor \Omega cassette DNA fragments exerts a polar effect on the expression of the essential downstream \textit{csmA} gene. Since the \Omega cassette contains strong transcription termination signals flanking the gene encoding drug resistance [16], these results suggest that at least some transcription of \textit{csmA} occurs from a promoter located between \textit{csmC} and \textit{csmA}. The 5’ endpoint of the major \textit{csmA} transcript of \textit{Cb. tepidum} has been mapped to a position 52 nucleotides upstream from the translational start site for this gene [8]. Thus, this endpoint may not arise from a processing event as originally suggested [8], and a strong promoter possibly occurs upstream from it.

Fig. 3. Immunoblot analysis of the wild-type and \textit{csmC} mutant strains of \textit{Cb. vibrioforme}. Whole cell extracts (proteins associated with 100 \mu g of Bchl \textit{d}) were separated by SDS-PAGE, electrophoretically transferred to Immobilon membranes (Millipore, Bedford, MA) and probed with polyclonal rabbit antibodies against the CsmC protein [7]. Lane 1, extract from wild-type cells; lane 2, extract from \textit{csmC} mutant strain 633; lane 3, extract from \textit{csmC} mutant strain 626.
To confirm that the \textit{csmC} mutant strains of \textit{Cb. vibrioforme} were missing CsmC, total proteins from whole cell extracts were separated by SDS-PAGE and immunoblotted with antibodies specific for CsmC. As indicated in Fig. 3, the immunoreactive 14.2-kDa CsmC protein observed in extracts of the wild-type cells (Fig. 3, lane 1) is missing extracts of mutant strains 633 (Fig. 3, lane 2) and 626 (Fig. 3, lane 3). Chlorosome proteins other than CsmC were verified to be present in approximately normal amounts by both SDS-PAGE and immunoblot analyses of isolated chlorosomes (data not shown).

To determine whether the absence of CsmC had any physiological effect on the growth rate of \textit{Cb. vibrioforme} strains 626 and 633, doubling times were monitored by optical density measurements at

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig5.png}
\caption{Fluorescence emission spectra at 77 K of whole cells and chlorosomes for the wild-type (solid lines) and \textit{csmC} mutant strain 633 (dashed lines) of \textit{Cb. vibrioforme}. The excitation wavelength was at 449 nm which is the maximum absorption peak of Bchl \textit{d} in the blue region of the spectrum (see Fig. 4). Spectra were measured at identical cell densities for whole cells (OD$_{650nm}$ = 0.2) and identical concentrations of Bchl \textit{d} (20 \text{ \micro g ml}^{-1} \text{}) for chlorosomes. A: Fluorescence emission spectra of whole cells. The two emission peaks occurred at 750 nm and 804 nm. B: Same as panel A but the spectra have been normalized at 804 nm. C: Fluorescence emission spectra of purified chlorosomes. The two emission peaks occurred at 750 nm and 800 nm.}
\end{figure}
Fig. 4A shows a comparison of the absorption spectra of whole cells of the wild-type and csmC mutant strain 633 of Chl. vibrioforme. As shown in Fig. 4A, the absorbance maximum of the csmC mutant strain in the near infrared region of the spectrum was shifted about 6 nm to the red compared to that of the wild-type. The absorption maximum of the wild-type strain occurred at 728 nm while that of the csmC mutant strain 633 occurred at 734 nm. However, the total Bchl content of the csmC mutant and wild-type cells were similar. In the absorption spectra of isolated chlorosomes (Fig. 4B), the absorbance maximum for the wild-type strain was 728 nm, but the absorbance maximum of chlorosomes isolated from csmC mutant strain 633 was shifted 2 nm to the red to 730 nm. These observations suggest that the loss of the CsmC polypeptide resulted in some changes in the environment or conformation of Bchl d in the chlorosomes.

To investigate the effect of the csmC mutation on energy transfer, steady-state fluorescence emission measurements were made at 77 K with whole cells (Fig. 5A,B) and isolated chlorosomes (Fig. 5C) and compared to the results obtained for the wild-type strain. Both the wild-type and csmC mutant strain 633 exhibited two emission peaks at about 750 nm and 804 nm. As shown in Fig. 5A, the steady-state fluorescence emission of the csmC mutant strain 633 was much greater than that from the wild-type, implying that energy transfer in chlorosomes might be impaired in the absence of CsmC. As seen in Fig. 5B,C, even when the fluorescence emission maxima of whole cells (Fig. 5B) or isolated chlorosomes (Fig. 5C) were normalized at the 804 nm maximum, the amplitude of the emission peak at about 750 nm was significantly greater for the csmC mutant than for the wild-type. The light-harvesting energy transfer pathway in Chl. vibrioforme is believed to be: antenna Bchl d (728 nm) → antenna Bchl a (794 nm) → baseplate Bchl a/FMO protein (808 nm) → reaction center Bchl a (840 nm) [1,2]. The cellular content, assembly and absorption properties of chlorosomes of the csmC mutant strain were not significantly different from those of wild-type cells (Fig. 4). Nevertheless, the absence of CsmC protein had demonstrable effects on the fluorescence emission properties of whole cells and isolated chlorosomes, and these data suggest that CsmC may play at least two roles in chlorosome function. Firstly, the fluorescence emission data for whole cells in Fig. 5A suggest that overall energy transfer from chlorosomes to the reaction centers is less efficient in the csmC mutant than in the wild-type strain. Thus, the absence of CsmC appears to affect the coupling of chlorosomes to the FMO protein and to reaction centers in some manner that causes total fluorescence emission at both 750 and 804 nm to increase substantially. Secondly, energy transfer from Bchl d to the species emitting at about 804 nm was also less efficient in the mutant than in the wild-type, as can be seen in the normalized spectra for whole cells (Fig. 5B) and isolated chlorosomes (Fig. 5C). Therefore, CsmC might also play a role in the organization of the bulk Bchl d, might be involved in the organization of the Bchl a of the chlorosome, or might be involved in the organization of some ‘special’ Bchl d molecules or oligomers that might serve as intermediary acceptors of light energy between the bulk Bchl d and Bchl a of chlorosomes. Although it is not possible at this time to distinguish among these possibilities, the effects on both the growth rate and the fluorescence emission properties are consistent with the idea that the absence of CsmC causes a decrease in the overall efficiency of light-energy harvesting. This in turn strongly supports the view that chlorosome proteins have specific and important roles in the structure and function of chlorosomes. Finally, the studies reported here establish the feasibility of using targeted mutagenesis to study the roles of proteins in the structure, function, and biogenesis of chlorosomes.

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References


