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Transformation of *Chlorobium limicola* by a Plasmid That Confers the Ability To Utilize Thiosulfate

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A modified transformation method for *Chlorobium limicola* confirms the role of an endogenous plasmid in thiosulfate metabolism. The plasmid was present in two forma specialis *thiosulfatophilum* (Tio^+) strains and absent in one non-thiosulfate-utilizing (Tio^-) strain. The plasmid (size, 14 kb) was transferred by transformation from Tio^- to Tio^+. The chromosomal restriction patterns, analyzed by pulsed-field gel electrophoresis, were used to distinguish between the different strains.

Although many physiological and ecological aspects of sulfur-utilizing phototrophic bacteria have been described, little is known about their genetics (2, 6, 12, 14) because of the long incubation period of the cultures and the lack of an easy, systematic, and quantitative method by which to obtain isolated colonies.

Green sulfur bacteria are anaerobic, photolithotrophic bacteria that require light as an energy source and suitable electron donors, such as hydrogen sulfide or elemental sulfur (3, 10). *Chlorobium* is the only genus that includes two species with forms able to utilize thiosulfate as a photosynthetic electron donor, *C. limicola* and *C. vibrioforme*. This capacity renders these forms easier to cultivate than those that depend on sulfide. Consequently, much more research has been carried out on these forms than on any others.

C. limicola includes two subspecies, *C. limicola* and *C. limicola* f. sp. *thiosulfatophilum* (9, 10). They basically differ in both their ability to utilize thiosulfate ($S_2O_3^{2-}$) and their molar G+C content. The *thiosulfatophilum* subspecies possesses high levels of thiosulfate sulfur transferase (rhodanese) (1, 16) and a cytochrome composition different from that of the non-thiosulfate-utilizing strains (5, 17). In spite of the fact that different aspects of the biochemistry of thiosulfate metabolism have been studied, nothing is known to date about the genetics of thiosulfate metabolism of these subspecies. Indeed, although natural genetic transformation in *Chlorobium* spp. has been reported (8), the lack of genetic tools available for the study of this group has been a hindrance for genetic mapping and manipulation of these microorganisms.

Materials. Three strains of the species *C. limicola* were used (Table 1). Liquid cultures of *C. limicola* were grown photolithoautotrophically in rubber-capped bottles in Pfennig minimal medium at pH 6.6 to 6.9 (18) to about 10^8 cells per ml. For colony growth, cultures were plated onto agar Pfennig minimal medium (18) and incubated in GasPak anaerobic jars (BBL, Cockeysville, Md.) supplemented with 0.10 g of thioacetamide (CH_3CSNH_2) dissolved in 1 ml of 0.35 N HCl, as described by Irgens (4). All cultures were performed at 23°C and under continuous illumination of 50 microeinsteins/m^2/s.

DNA preparation and restriction enzyme digestion. Intact genomic DNAs were prepared in agarose plugs as described by Smith and Cantor (15). Transforming DNA was purified by the alkaline method for mini preparation of plasmidic DNA (11), routinely used to isolate plasmids smaller than 50 kb. Restriction assays were carried out as described previously (7, 11). Restriction enzymes and DNA standards were purchased from New England Biolabs (Beverly, Mass.) and Boehringer Mannheim (Mannheim, Germany).

Pulsed-field gel electrophoreses. Pulsed-field gel electrophoreses (13) were performed in a Pharmacia-LKB (Uppsala, Sweden) apparatus. Gels were made of 1% agarose (SeaKem LE agarose; FMC, Rockland, Maine) and run at 15°C in modified TBE buffer (100 mM Tris, 100 mM boric acid, 0.2 mM EDTA; the final pH was 8 to 8.4).

Isolation of a 14-kb plasmid from forma specialis *thiosulfatophilum* strains. Conventional methods were applied to detect plasmids smaller than 50 kb (11). A 14-kb plasmid was detected in the two thiosulfate-utilizing strains analyzed, *C. limicola* DSM 249 and BF 8000. The isolated plasmid was cleaved with the restriction endonucleases *Eco*RV and *Hin*dIII and then subjected to conventional electrophoresis; two bands of 7.5 and 6.5 kb were identified when it was cleaved with *Eco*RV (Fig. 1); five bands of 6, 4, 2.1, 1.1, and 0.8 kb were identified when it was cleaved with *Hin*dIII (data not shown). The presence of this plasmid in the two strains able to grow with thiosulfate (Tio^+), along with its absence in the non-*thiosulfatophilum* strain (Tio^-), suggests that it might determine some functions related to thiosulfate metabolism. To test this possibility, a transformation method for *C. limicola* was developed and the strain DSM 245 (Tio^-) was transformed with the plasmid.

Transformation of *C. limicola* DSM 245. *C. limicola* DSM 245 (Tio^-) was transformed with a naturally occurring plasmid isolated from *C. limicola* DSM 249 (Tio^+) by the method described by Sambrook et al. (11), modified in order to preserve anaerobic conditions. A culture of the recipient strain was grown in minimal medium to the end of the log phase, and cells were then harvested and resuspended in a solution of 50 mM $CaCl_2$ (10% initial volume), which had been saturated with a mixture of 95% N_2 and 5% CO_2 while being stirred magnetically. A 0.2-ml portion of this suspension was treated with 100 ng of transforming DNA, as described by Sambrook et al. (11). Following heat shock at 40°C, the DNA-cell mixture was added to 1 ml of minimal medium and incubated overnight inside an anaerobic bag (BBL) at 23°C under continuous

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TABLE 1. Bacterial strains[a]

Strain	Source	Thiosulfate utilization	G+C content (%)
C. limicola	DSM[b]	−	49.0
C. limicola f. sp. *thiosulfatophilum*	DSM	+	58.1
C. limicola BF 8000[c]	UAB[d]	+	ND

[a] −, negative; +, positive; ND, not determined.
[b] DSM, Deutsche SammTung von Mikroorganismen.
[c] Strain isolated from Lake Cisó (Girona, Spain) by M. Algueró.
[d] UAB, Universitat Autònoma de Barcelona.

illumination before being plated onto minimal medium supplemented with sodium thiosulfate at a final concentration of 5 mM. In order to ensure bacterial growth, the transformants were selected in two consecutive steps. Firstly, plates were incubated for 2 weeks in GasPak anaerobic jars supplemented with 0.10 g of thioacetamide dissolved in 1 ml of 0.35 N HCl, as described above. After this step, *C. limicola* DSM 245 (Tio^-) showed a residual growth, but colony growth was only observed when *C. limicola* DSM 245 (Tio^-) was transformed with the isolated plasmid and consequently acquired the ability to utilize thiosulfate. These colonies were comparable to those of *C. limicola* f. sp. *thiosulfatophilum* DSM 249. Secondly, colonies were again plated on minimal medium supplemented with 5 mM thiosulfate and incubated in GasPak anaerobic jars without thioacetamide. Under these culture conditions, only cells able to utilize thiosulfate as the only electron donor for photosynthesis can grow. *C. limicola* f. sp. *thiosulfatophilum* DSM 249 and the transformants were able to grow, while *C. limicola* DSM 245 (Tio^-) did not grow, which suggested that the plasmid conferred the capability to metabolize thiosulfate. The efficiency of the transformants was 1×10^{-4} to 2×10^{-4} transformants per μg of DNA.

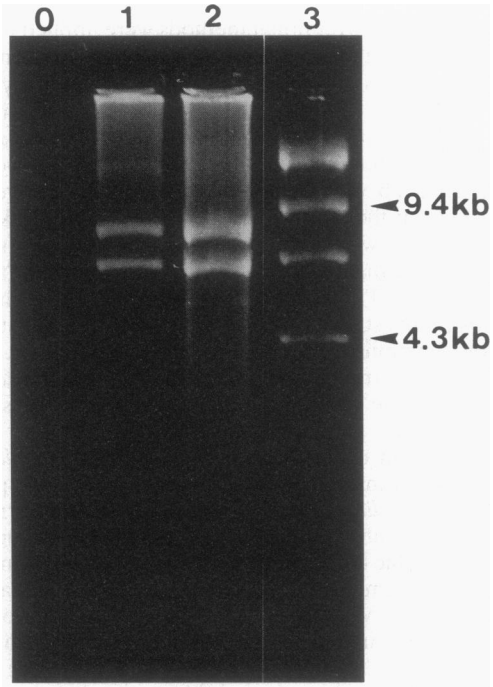

FIG. 1. Agarose gel electrophoresis of *Eco*RV restriction patterns of plasmids isolated from *C. limicola* DSM 249 (lane 2) and *C. limicola* DSM 245 transformants (lane 1). The negative control was *C. limicola* DSM 245 (lane 0). The molecular weight marker was lambda DNA digested with *Hin*dIII (lane 3).

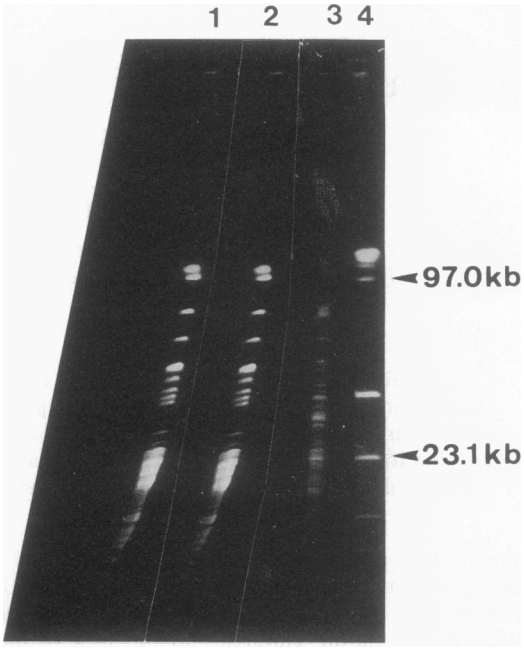

FIG. 2. Comparison of *Ase*I restriction patterns from *C. limicola* DSM 249 (lane 1) and DSM 245 (lane 2) and *C. limicola* DSM 245 transformants (lane 3). The molecular weight marker was lambda concatemers plus lambda DNA digested with *Hin*dIII (lane 4).

When *C. limicola* DSM 245 transformants were grown on minimal medium (3 mM sulfide), supplemented with thiosulfate at a final concentration of 5 mM, they showed a growth rate similar to that of *C. limicola* f. sp. *thiosulfatophilum* DSM 249, while *C. limicola* DSM 245 (Tio^-) showed a residual growth due to the presence of sulfide in the medium. Control flasks containing minimal medium without any thiosulfate did not show significant differences between the growth of *thiosulfatophilum* strains, that of transformants, and that of *C. limicola* DSM 245 (data not shown).

Plasmids isolated from transformants. Plasmid DNA isolated from the *C. limicola* DSM 245 transformants matched the *C. limicola* DSM 249 plasmid DNA in both size and *Eco*RV and *Hin*dIII restriction patterns (Fig. 1).

Transformant identification. The only phenotypic difference between the two subspecies *C. limicola* DSM 249 and *C. limicola* DSM 245 is the ability of the former to utilize thiosulfate, which made it impossible to distinguish, through phenotypic characters, the transformants from the donor strain. Because of this impossibility, they were distinguished by analyzing their genetic differences. Pulsed-field gel electrophoresis was thus combined with the use of restriction endonucleases in order to use chromosomal restriction patterns as an identifying criterion, as they had been used with other sulfur phototrophic bacteria by Gaju et al. (2a). For this purpose, various restriction endonucleases were assayed: *Ase*I, *Bcl*I, *Dra*I, *Eco*RV, and *Sfi*I. The restriction endonuclease *Ase*I, which generated fragments with sizes between 6 and 100 kb, was used, since it yielded the best resolution. Figure 2 shows that transformants had the same restriction pattern as *C. limicola* DSM 245 (Tio^-), which confirms the theory that the

plasmid isolated from the *thiosulfatophilum* strain DSM 249 conferred on *C. limicola* DSM 245 (Tio^-) the ability to metabolize thiosulfate.

Concluding remarks. These results reveal that the isolated plasmid probably codes for the ability to use thiosulfate or for some regulator required for activation or increased expression of silent thiosulfate genes. However, further research is needed to elucidate its exact role in such metabolism and the different genes involved in the metabolism.

To our knowledge, the transformation system described here for the phototrophic bacterium *C. limicola* is the first method successfully used to make cells from this species competent for natural isolated plasmid DNA uptake by exposing harvested cells to $CaCl_2$. The development of an easy transformation method for *Chlorobium* spp. satisfies a need to increase the genetic transfer systems of the green sulfur bacteria. Moreover, the plasmid described is not only an important genetic element from the point of view of thiosulfate metabolism, but it constitutes a potential cloning vector for genetic manipulation in *C. limicola*. In fact, it provides a genetic marker, the ability to utilize thiosulfate. In summary, its simplicity, the lack of any difficulty in preserving anaerobic conditions throughout all the process, and the availability of a potential vector which codes for an effective genetic marker suggest that this method could be more versatile, more efficient, and easier to carry out with *Chlorobium* spp. than other methods currently used with other microorganisms. Besides, pulsed-field gel electrophoresis, combined with the use of restriction endonucleases, seems to be an efficient tool for the identification of different strains of green sulfur bacteria.

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