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Use of Electroporation To Generate a *Thiobacillus neapolitanus* Carboxysome Mutant

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Two cloning vectors designed for use in *Escherichia coli* and the thiobacilli were constructed by combining a *Thiobacillus intermedius* plasmid replicon with a multicloning site, *lacZ′*, and either a kanamycin or a streptomycin resistance gene. Conditions necessary for the introduction of DNA into *T. intermedius* and *T. neapolitanus* via electroporation were examined and optimized. By using optimal electroporation conditions, the gene encoding a carboxysome shell protein, *csoS1A*, was insertionally inactivated in *T. neapolitanus*. The mutant showed a reduced number of carboxysomes and an increased level of CO_2 necessary for growth.

The thiobacilli are gram-negative, chemolithotrophic bacteria which derive their carbon from carbon dioxide and energy from the oxidation of reduced sulfur compounds. These aerobic bacteria play an essential role in the environmental sulfur cycle (2), have been implicated in concrete corrosion (22) and acidification of mine tailings (2), and are used industrially in the bioleaching of low-grade ores (23, 36). Recent advances in the establishment of a genetic system for *Thiobacillus ferrooxidans* were prompted by its use in industrial applications. Included in these achievements are the construction of shuttle vectors (29, 30) and the development of electroporation (18) and conjugation methodologies (24). For a recent review, see reference 28.

The establishment of a genetic system for the other thiobacilli has progressed more slowly. Conjugation from *Escherichia coli* into the neutrophilic bacterium *T. neapolitanus* was first demonstrated by using the IncP broad-host-range plasmid RP1 (17). Another group, however, was unable to conjugate into *T. neapolitanus* directly from *E. coli*. By using *T. novellus* as an intermediary, they were able to transfer DNA into *T. neapolitanus*, *T. intermedius*, *T. acidophilus*, *T. perometabolis*, and *T. versutus* (7, 8). Conjugation into the acidophilic bacterium *T. thiooxidans* has also been demonstrated (15).

The biochemistry of carbon metabolism in the thiobacilli is an area of ongoing research (4, 5, 10, 19, 31–35). Growing autotrophically, *T. neapolitanus* and *T. intermedius* utilize the initial enzyme of the Calvin cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), for CO_2 fixation (27, 31). Like many autotrophic bacteria, these thiobacilli are able to sequester RuBisCO into polyhedral inclusion bodies called carboxysomes (6). Carboxysomal RuBisCO appears to be more efficient in CO_2 fixation; however, the exact mechanism by which carboxysomes aid in CO_2 fixation is unknown. The major carboxysome shell peptide gene from the strict autotroph *T. neapolitanus* has also been isolated and sequenced (10). Inactivation of this gene in *T. neapolitanus* is desirable to confirm not only the role of this peptide in carboxysome structure but also the importance of the carboxysome in supplying fixed carbon to the cell. To accomplish this, it was essential that we establish the optimal conditions for introducing DNA into *T. neapolitanus* and *T. intermedius*. In addition, it was necessary that we determine which cloning vectors would be useful in these thiobacilli. Herein, we describe the incorporation of the replicon from a *T. intermedius* native plasmid into cloning vectors which are stable in *T. neapolitanus* and the conditions necessary for introduction of DNA into both *T. neapolitanus* and *T. intermedius* via electroporation. By using the optimal electroporation conditions, we were able to generate a *T. neapolitanus* carboxysome mutant.

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MATERIALS AND METHODS

Cultures. *T. intermedius* K12 (from E. Bock, University of Hamburg, Hamburg, Germany) was grown in autotrophic medium containing (per liter) 0.4 g of KH_2PO_4, 0.6 g of K_2HPO_4, 1 g of NH_4Cl, 0.13 g of $CaCl_2 \cdot 2H_2O$, 0.498 g of $MgSO_4 \cdot 7H_2O$, 5 g of $Na_2S_2O_3$, 5 ml of Fe-EDTA (0.4 g of Na_2 EDTA per liter, 0.2 g of $FeCl_3 \cdot 6H_2O$ per liter), 5 ml of trace elements (37), and 2.5 mg of biotin. *T. neapolitanus* ATCC 23641 was grown in the medium of Vishniac and Santer (37) with thiosulfate as the energy source. To ensure a reproducible physiological state of cells in electroporation experiments, both organisms were cultivated in chemostats at 30°C. The high-CO_2-requiring mutant of *T. neapolitanus* was grown in an atmosphere supplemented with 5% CO_2.

E. coli DH5α served as the host strain for plasmid manipulations and was grown at 37°C in Luria-Bertani medium (20). Antibiotics were added as required for *E. coli* at the following concentrations: kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; ampicillin, 100 μg/ml. For the thiobacilli, antibiotic concentrations were as follows: kanamycin, 10 μg/ml; ampicillin, 25 μg/ml.

Electroporation of thiobacilli. *T. intermedius* was grown in a 1-liter chemostat with the pH maintained at 5.5. *T. neapolitanus* was grown in a 2-liter chemostat with the pH maintained at 6.7. Both chemostats were thiosulfate limited at a dilution rate of 0.08 h^{-1}. Cells from the *T. intermedius* chemostat were collected in a 2-liter flask on ice, while *T. neapolitanus* cells were collected directly from the chemostat. Cells were harvested by centrifugation, resuspended, recentrifuged twice at 4°C with an equal volume of cold Nanopure water, and then resuspended in 0.01 volume of cold water. Electrocompetent cells (50 μl) were mixed with 2 μl of plasmid DNA (1 μg) and transferred into a prechilled electroporation cuvette with a 0.1-cm gap. The pulses were generated at room temperature (200 Ω of resistance, 25 μF of capacitance) by a Gene Pulser apparatus with a pulse controller (Bio-Rad Laboratories, Richmond, Calif.). After electroporation, the cell-DNA mixture was transferred immediately to 5 ml of cold liquid medium and kept on ice for 5 min. The cell suspension was incubated with shaking at 30°C to allow expression of the antibiotic resistance gene before plating.

Cloning techniques. The general molecular cloning techniques used, such as plasmid isolation, restriction digests, and agarose gel electrophoresis, were those described by Maniatis et al. (20). Transformations were accomplished by using the method of Hanahan (14). Ligations with T4 DNA ligase were done by following the manufacturer's recommendations (Bethesda Research Laboratories, Gaithersburg, Md.). Isolation of restriction fragments from agarose gels was accomplished by electroelution followed by purification with an Elutip column (Schleicher & Schuell, Inc., Keene, N.H.). Southern blotting, probe preparation, and hybridization were accomplished as previously described (32).

Construction of cloning vectors. Because kanamycin is preferable to tetracycline selection in thiobacilli, the broad-host-range IncP plasmid pRK415 (16) was altered by the insertion of a 1.2-kbp *Pst*I fragment containing a kanamycin resistance gene to create pRK415Km. Two vectors based upon the previously described *T. intermedius* plasmid replicon were constructed (9) (Fig. 1).

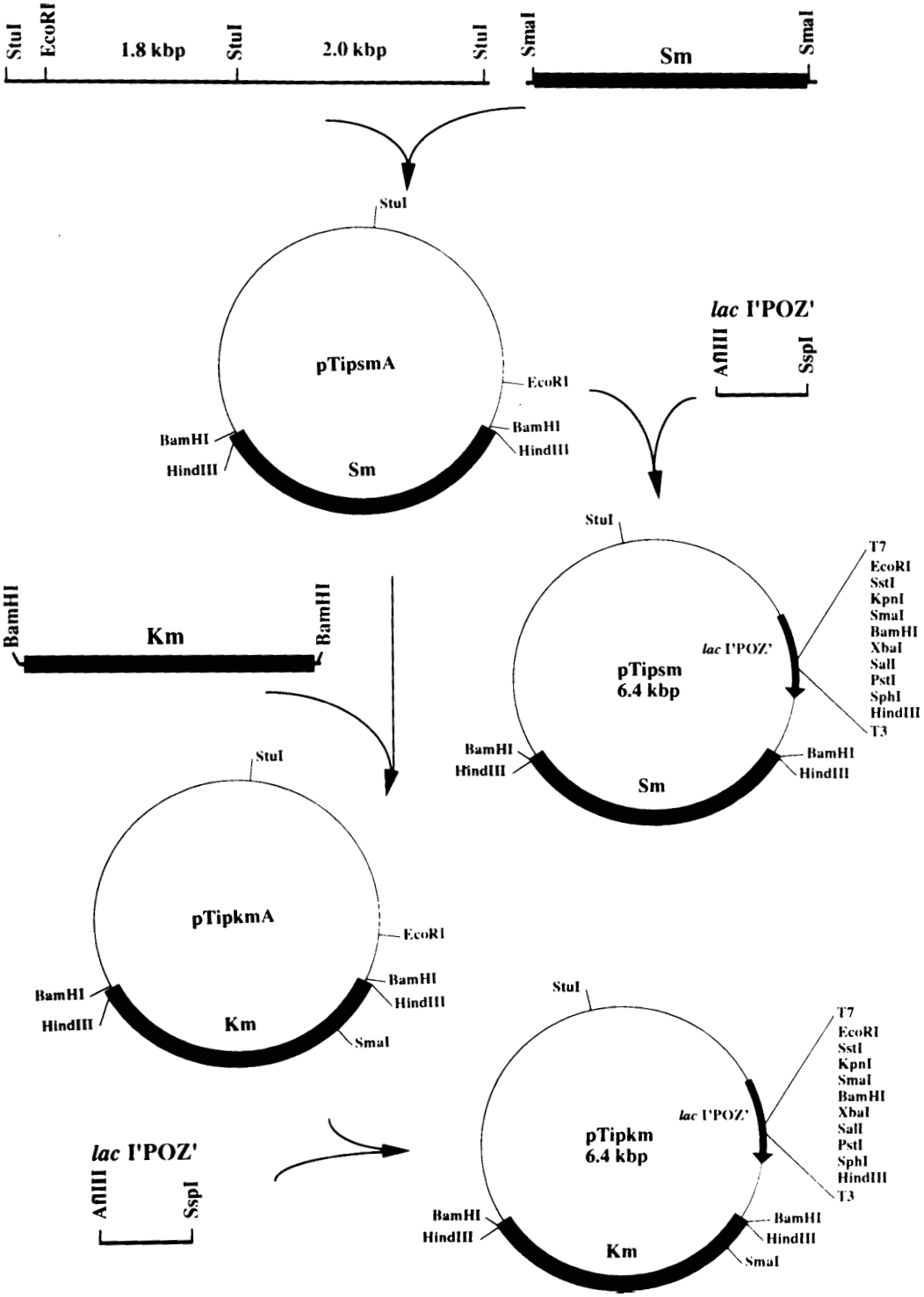

FIG. 1. Construction of cloning vectors. Two *Stu*I fragments containing the *T. intermedius* plasmid origin of replication were ligated to an *Sma*I-digested streptomycin resistance cartridge (from pHP45Ω [11]) to create pTipsmA, which is able to replicate in *E. coli*. This construct was digested with *Eco*RI, and the ends were made blunt with mung bean nuclease (United States Biochemical Corp., Cleveland, Ohio). The region containing *lacI'POZ'* from pT7/T3α18 (Bethesda Research Laboratories) was removed by digestion with *Ssp*I and *Afl*III. The recessed end of the *Afl*III site was made blunt with mung bean nuclease. This fragment, carrying the *lac* region, was ligated to *Eco*RI-digested (blunt-ended) pTipsmA to give pTipsm. pTipkm was constructed by removing the streptomycin resistance cartridge from pTipsmA with *Bam*HI and replacing it with a *Bam*HI-digested kanamycin resistance cartridge from pHP45Ωkm (11). Like pTipsm, pTipkm was constructed by insertion of the *lac* region into the *Eco*RI site of pTipkmA.

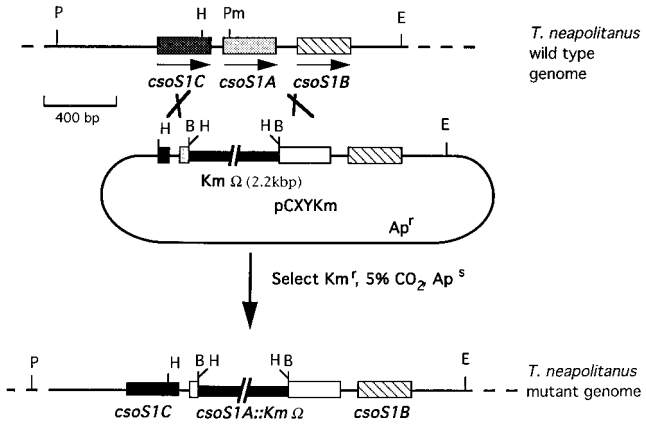

FIG. 2. Inactivation of the *T. neapolitanus* carboxysome gene. Suicide plasmid pCXYKm, containing the interrupted *csoS1A* gene, was able to replace the genomic copy of *csoS1A* through homologous recombination. Selection for the desired double recombinant was based upon resistance to kanamycin, sensitivity to ampicillin, and growth with 5% CO_2. Restriction enzymes are abbreviated as follows: *Pst*I, P; *Hin*dIII, H; *Bam*HI, B; *Eco*RI, E; *Pml*I, Pm.

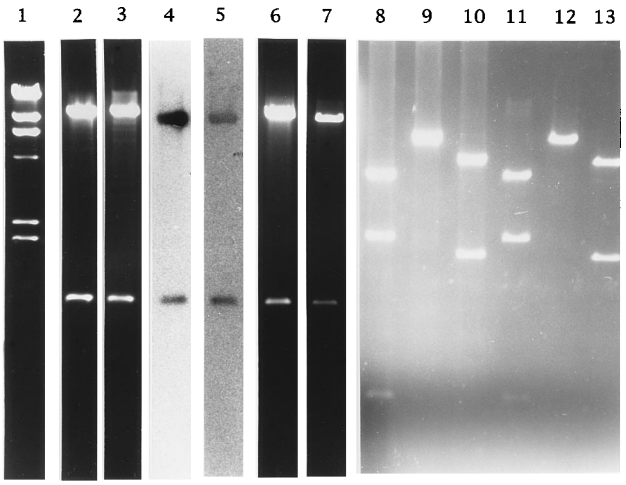

FIG. 3. Plasmid DNAs from electroporated *T. intermedius* and *T. neapolitanus*. Lane 1 contained *Hin*dIII-digested lambda DNA as a size marker. Lanes 2 and 3 contained pRK415Km from *E. coli* and *T. intermedius*, respectively, digested with *Pst*I. Lanes 4 and 5 are the accompanying autoradiographs from the Southern blot of the previous lanes hybridized with pRK415Km. Lanes 6 and 7 contained pRK415Km from *E. coli* and *T. neapolitanus*, respectively, digested with *Pst*I. Lanes 8, 9, and 10 contained pTipKm from *E. coli* digested with *Bam*HI, *Eco*RI, and *Eco*RI-*Stu*I, respectively. Lanes 11, 12, and 13 contained the same digests of pTipkm from *T. neapolitanus*.

To measure the stability of plasmids in thiobacilli, plasmid-containing cultures were transferred for 50 generations in liquid medium either with or without selective pressure. Cells were then plated on nonselective medium. One hundred colonies each were transferred to selective and nonselective plates and scored for growth.

Generation of a *T. neapolitanus* carboxysome mutant. The gene coding for the *T. neapolitanus* carboxysome shell protein (*csoS1A*) (10) was inactivated by disruption of the gene with an omega fragment (Fig. 2). The 1.3-kbp *Hin*dIII-*Eco*RI fragment containing the *csoS1AB* genes was cloned into pT7/T3α18. It was determined that pUC-derived plasmids were unable to replicate in *T. neapolitanus* and could be used as suicide vectors (data not shown). An omega fragment (11, 25) containing the resistance gene for kanamycin was introduced into a *Pml*I site 40 bp within *csoS1A* by converting the blunt site into a *Bam*HI site through addition of linkers attached to a streptomycin resistance gene. The streptomycin resistance gene was removed by digestion with *Bam*HI and replaced with the kanamycin resistance omega fragment. Optimal electroporation parameters were used to introduce this construct into *T. neapolitanus*. After electroporation, cells were recovered overnight with 5% CO_2, kanamycin was added to 50 μg/ml, and recovery continued for 24 h before plating. Colonies were screened for resistance to kanamycin, sensitivity to ampicillin (marker on the suicide vector), and lack of growth at atmospheric levels of CO_2.

RuBisCO enzyme assay and determination of the soluble/carboxysomal RuBisCO ratio. Ribulose bisphosphate-dependent assays were done at room temperature with $NaH[^{14}C]O_3$ as described by Shively et al. (31). The ratio of carboxysomal to free RuBisCO was determined by differential centrifugation and enzyme assays after cell disruption by sonication. This method has been previously established for *T. neapolitanus* and confirmed by electron microscopy (1, 3).

RESULTS

Electroporation of thiobacilli. Both *T. neapolitanus* and *T. intermedius* were able to be electroporated (Fig. 3). Broad-host-range plasmid pRK415Km and thiobacillus-derived replicon pTipkm were introduced into *T. neapolitanus* and were maintained with antibiotic pressure. Without antibiotic pressure, pTipkm was more stable than pRK415Km. Fifty-nine percent of the pTipkm colonies and 0% of the pRK415Km colonies tested remained resistant after 50 generations of growth without an antibiotic. A larger *T. intermedius* plasmid replicon (which has a lower copy number), pTip11.5 (9), was stable in 100% of the colonies tested, even in the absence of selective pressure. In *T. intermedius*, pRK415Km was maintained with selective pressure but was unstable without antibiotic selection (0% of colonies tested). When pTipkm (or pTip11.5) was introduced into *T. intermedius*, transformants could be isolated from plates but did not survive subculturing. Possibly, the native 65-kbp plasmid from which these vectors were derived is required for growth and is not compatible with the pTip plasmids.

Effects of various parameters upon electroporation efficiency. To use electroporation as a practical tool for introducing DNA into thiobacilli, it was necessary to determine the optimal electroporation conditions. With pRK415Km as the source DNA, several parameters were tested. The field strength used during electroporation was the single most critical parameter examined. The optimal field strength for *T. intermedius* was 20 kV/cm, while for *T. neapolitanus* it was 19 kV/cm. Both optimal field strengths resulted in approximately 30% cell survival.

The optimal amount of DNA to use in electroporation was determined by varying the amount of plasmid DNA used from 0.5 to 2.5 μg. Different amounts of pRK415Km in a total volume of 2 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were used to electroporate both *T. intermedius* and *T. neapolitanus*. The efficiency of electroporation (number of transformants per microgram of DNA) decreased linearly as the amount of DNA used increased (data not shown). With this protocol, the total number of transformants, for both *T. intermedius* and *T. neapolitanus*, was optimal with approximately 1.5 μg of DNA.

The effect of plasmid volume was also examined with respect to electroporation efficiency with *T. intermedius*. Plasmid DNA (1 μg) in volumes of TE buffer ranging from 1 to 30 μl was added to 50 μl of electrocompetent cells and electroporated. The efficiency drastically decreased as the volume was increased (data not shown). For example, the number of transformants dropped from 6.8×10^3 CFU with a 1-μl volume to 5.0×10^2 CFU with a 5-μl volume. This is indicative of the need for high concentrations of both cells and DNA for optimal efficiency.

Some researchers have reported that incubation of the plasmid DNA-cell mixture on ice prior to electroporation increases efficiency. In our study, no difference in electroporation efficiency was detected during a 10-min preincubation period. In

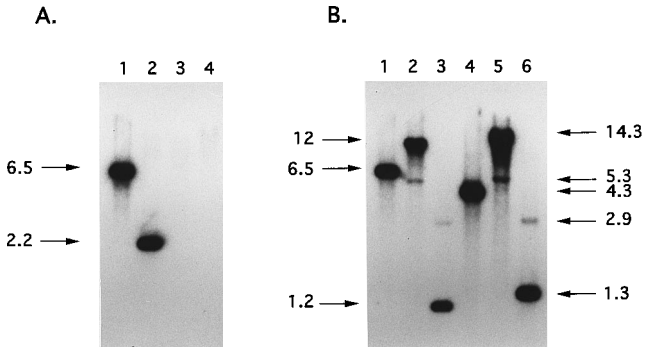

FIG. 4. Confirmation of the recombinational event. (A) Autoradiograph resulting from hybridization with the kanamycin resistance Ω fragment. Lanes 1 and 2 contained *T. neapolitanus csoS1A*::KmΩ genomic DNA digested with *Eco*RI and *Bam*HI, respectively. Lanes 3 and 4 contained *T. neapolitanus* wild-type genomic DNA digested with *Eco*RI and *Bam*HI, respectively. The sizes of the restriction fragments are indicated on the side in kilobase pairs. (B) Autoradiograph resulting from hybridization with *csoS1A*. Lanes 1, 2, and 3 contained *T. neapolitanus csoS1A*::KmΩ genomic DNA digested with *Eco*RI, *Hin*dIII, and *Eco*RI-*Hin*dIII, respectively. Lanes 4, 5, and 6 contained *T. neapolitanus* wild-type genomic DNA digested with *Eco*RI, *Hin*dIII, and *Eco*RI-*Hin*dIII, respectively. The sizes of the restriction fragments are indicated on the sides in kilobase pairs. The weak signal observed at 5.3 and 2.9 kbp resulted from cross-hybridization of *csoS1A* with *csoS1C*.

fact, decreased electroporation efficiency was observed after 10 min, possibly because of degradation of the plasmid DNA.

The optimal time of recovery in liquid medium prior to plating was determined for *T. neapolitanus* to be 4 h, which corresponds to approximately 1 doubling time.

Characterization of the *csoS1A* mutant. To confirm the function of CsoS1A, a mutant was constructed in which the gene was interrupted by an interposon (Fig. 2 and 4). Growth curves of the mutant show that high levels of CO_2 are required and are able to compensate for the carboxysome mutation (Fig. 5). To examine the amount of RuBisCO packaged inside carboxysomes relative to the amount of free enzyme, the ratios of

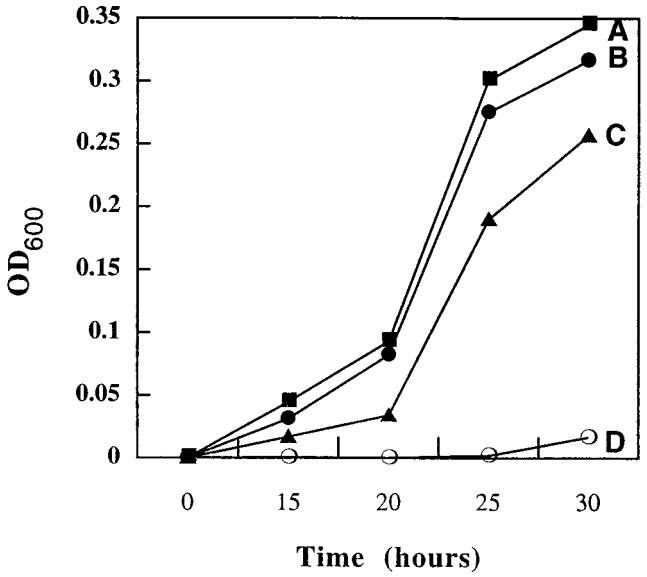

FIG. 5. *T. neapolitanus csoS1A*::KmΩ and *T. neapolitanus* wild-type growth curves. (A) *T. neapolitanus* wild type under 5% CO_2. (B) *T. neapolitanus csoS1A*::KmΩ under 5% CO_2. (C) *T. neapolitanus* wild type under air. (D) *T. neapolitanus csoS1A*::KmΩ under air. OD_{600}, optical density at 600 nm.

TABLE 1. Soluble and particulate RuBisCO activities

T. neapolitanus strain	RuBisCO activity[a]		Soluble/particulate activity ratio
	Soluble	Particulate	
Wild type			
In air	2,480	1,158	2.14
With 5% CO_2	820	336	2.44
csoS1A::KmΩ			
In air	1,775	221	8.03
With 5% CO_2	1,145	176	6.51

[a] Total activity per standard volume reported as nanomoles of CO_2 fixed per minute.

soluble (free enzyme) to particulate (carboxysomal) RuBisCO activity were determined for the mutant and wild type (Table 1). Note that for determining the soluble to particulate enzyme ratios, RuBisCO activities were not adjusted for protein concentration and therefore the total amounts of enzyme activity in the two strains are not comparable. A reduced number of carboxysomes per cell was observed by electron microscopy, but no attempt was made to quantify this observation.

DISCUSSION

Utility of cloning vectors. Two vectors based upon the sequenced 3.8-kbp *T. intermedius* plasmid replicon (9), pTipsm and pTipkm, were constructed (Fig. 1). These plasmids stably replicate in *E. coli* and *T. neapolitanus*, maintain many of the unique sites in the pT7/T3α18 multicloning site, allow blue-white screening by α complementation in *E. coli*, and contain markers which are suitable for selection in thiobacilli. Vectors incorporating the *T. intermedius* replicon are more stable than pRK415 in *T. neapolitanus*. Unfortunately, the apparent requirement for the larger native plasmid in *T. intermedius* does not allow the pTip vectors to be used in *T. intermedius*.

Optimization of electroporation parameters. While examining factors affecting electroporation, it was noticed that increasing amounts of DNA seemed to decrease the efficiency of electroporation. Above approximately 1.5 μg of DNA, the conductance of the cell-DNA mixture was increased because of the ability of the DNA to act as a conductor. The increased conductance at higher DNA concentrations decreased the actual time of the electroporation pulse and therefore resulted in fewer transformants.

Carboxysome mutant. The optimization of DNA introduction into *T. intermedius* and *T. neapolitanus* via electroporation gave us the opportunity to examine gene function by generating a specific null mutant. The gene encoding the major carboxysome shell peptide of *T. neapolitanus* was inactivated. The high CO_2 requirement for growth and the shift from carboxysomal (particulate) to free (soluble) RuBisCO demonstrate the essential role of this peptide in proper carboxysome formation and, ultimately, in efficient CO_2 fixation. The observation that the *csoS1A* mutant still forms some carboxysomes demonstrates the apparent ability of CsoS1C (>90% identical to CsoS1A) to partially compensate for the lack of a major shell protein. While this heterologous complementation allows the formation of a few carboxysomes, it does not allow the mutant to grow at atmospheric levels of CO_2.

The high-CO_2-requiring phenotype of this mutant appears to be very similar to that of putative *ccmK*, *ccmL*, *ccmM*, *ccmN*, and *ccmO* carboxysome mutants of the cyanobacterium *Synechococcus* strain PCC 7942 (12, 13, 21, 26). There is a high degree of amino acid sequence conservation among CsoS1A,

CcmK, and CcmO (10). The results presented here demonstrate that a carboxysome shell mutant does result in a requirement for CO_2 supplementation, which is consistent with the contention that these *Synechococcus* open reading frames are involved in proper carboxysome structure-assembly.

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