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Organization of Carboxysome Genes in the Thiobacilli

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Abstract. The order of genes in the carboxysome gene clusters of four thiobacilli was examined and the possibility of the cluster forming an operon evaluated. Furthermore, carboxysome peptide homologs were compared with respect to similarities in primary sequence, and the unique structural features of the shell protein CsoS2 were described.

Carboxysomes, present in several different chemoautotrophic bacteria and in all cyanobacteria, are simple "organelles" or microcompartments that enhance the fixation of carbon dioxide by ribulose bisphosphate carboxylase/oxygenase (RuBisCO). The polyhedral carboxysomes are surrounded by a monolayer proteinaceous shell and appear to be filled with RuBisCO (reviewed in [7]).

The carboxysomes of *Halothiobacillus neapolitanus* ATCC 23641 [7] are composed of at least the eight peptides CbbL, CbbS, CsoS1A, CsoS1B, CsoS1C, CsoS2A, CsoS2B, and CsoS3, all of which are encoded by genes in a putative carboxysome operon [3, 10, 20, 23, 24]. The genes of the putative operon, in their transcriptional order, are *cbbL*, *cbbS*, *csoS2*, *csoS3*, *orfA*, *orfB*, *csoS1C*, *csoS1A*, *csoS1B* [23, 24]. The gene *csoS2* codes for the two carboxysome peptides CsoS2A and CsoS2B that are distinguished by differing degrees of post-translational glycosylation [3]. The CsoS1A, CsoS1B, CsoS1C, CsoS2A, CsoS2B, and CsoS3 proteins are all localized in the shell, with the CsoS1 peptide constituting the major component [3, 4, 6, 10]. Genes *csoS1A*, *csoS1B*, *csoS1C*, and *orfA* and *orfB* represent three- and two-gene repeats, respectively. Location in the carboxysome and function of the peptides encoded by *orfA* and *orfB* have yet to be elucidated. If these peptides are, indeed, structural components of the carboxysome, they are likely present in a low, to this point undetectable, concentration. Alternatively, these peptides might be essential for the formation/assembly, rather than the structural integrity of the carboxysome.

Herein we report on the organization of the carboxysome genes in three additional sulfur bacteria (thiobacilli), *Thiobacillus denitrificans* ATCC 25259, *Thiomonas intermedia* K12 [17], and *Acidithiobacillus ferrooxidans* ATCC 23270 [14]. The relationship between the carboxysome genes of the thiobacilli, those of the cyanobacteria [13, 16, 19], and the polyhedral bodies of enteric bacteria [5, 15] have been introduced elsewhere [7, 25, 26] and will not be covered in this report.

Materials and Methods

Culturing. The culturing and DNA isolation have been reported for *Tb. denitrificans* ATCC 25259 [9], *Th. intermedia* K12 [27, 28], and *A. ferrooxidans* ATCC 23270 [10].

Cloning and analysis of carboxysome gene order. Screening a *Tb. denitrificans* pLAFR5 cosmid library with *cbbL* of *Anacystis nidulans* and *cbbM* of *Rhodospirillum rubrum* [22] resulted in the isolation of three clones [10]. Clone pTdF12 contains *cbbL*/*cbbS* and *cbbM*, whereas pTdFI harbors *cbbL*/*cbbs*, and pTdF2 encompasses *cbbM*. By probing these clones with the *csoS1A* gene of *H. neapolitanus*, pTdF2 and pTdF12 were both found to also contain *csoS*1 genes (unpublished). Restriction mapping allowed the preparation of five subclones from *Eco*RI, *Kpn*I, and *Sma*I digests of pTdF2 by using the vector pT7/T3α18. The cloned *Tb. denitrificans* DNA fragments of the result-

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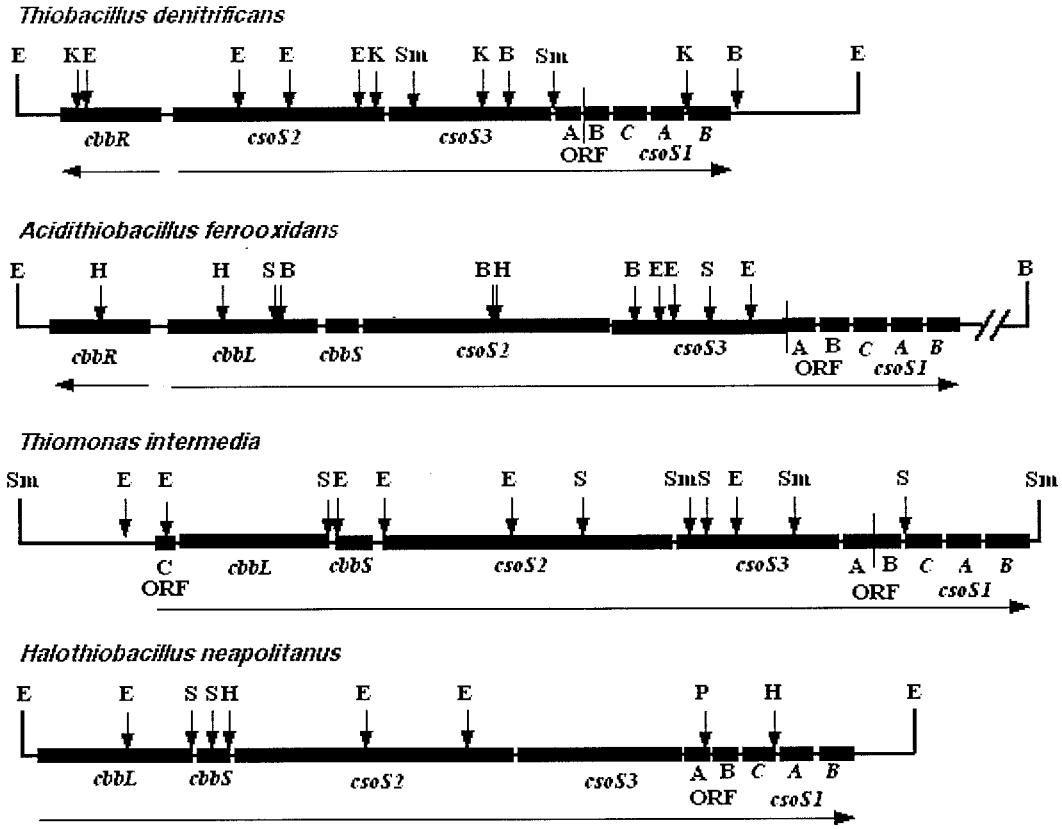

Fig. 1. Comparison of the carboxysomal gene order in four thiobacilli. The black boxes denote the individual genes. Horizontal arrows indicate direction of transcription. Letters with vertical downward arrows mark restriction sites for *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Pst*I (P), *Sal*I (S), and *Sma*I (Sm). The vertical lines in the clusters of *T. denitrificans* and *H. neapolitanus* are used to separate genes when they are very close together or overlap.

ing pTdF2E, pTdcsoE, pTd8S, pTd2.9K, and pTd7.5K encompass the form II RuBisCO gene *cbbM* and potentially all of the putative carboxysome operon, as revealed by preliminary sequence analysis. These subclones, along with pTdF2, were used for sequencing.

The isolation of pAfcbbLS1, which encodes both the large and small subunits of RuBisCO from the genomic DNA of *A. ferrooxidans* has been reported [11]. This clone did not show homology with the *csoS1A* gene of *H. neapolitanus*. Probing Southern blots of restriction-digested genomic DNA of *A. ferrooxidans* with *csoS1A* of *H. neapolitanus* demonstrated that *csoS1* resides on a 10-kbp *Hin*dIII fragment in *A. ferrooxidans*. This fragment was isolated by the methodology described previously [11] and yielded clone pAfcsoS1H. To determine the placement of the RuBisCO genes in relation to carboxysome genes, pAfcbbLS1 and pAfcsoS1H were digested with *Eco*RI and *Hin*dIII and resolved on a 0.8% agarose gel. A 1.5-kbp *Eco*RI/*Hin*dIII fragment appeared to be common to both recombinant plasmids. Sequencing these clones confirmed that this DNA fragment from both plasmids was identical and resolved the gene order of the carboxysome cluster in this bacterium.

Screening a *Tm. intermedia* K12 *Sau*3A-lambda library with the *cbbL* gene from *A. nidulans* resulted in the isolation of three positive clones designated *Ti102*, *Ti104*, and *Ti108* [27, 28]. Probing these clones with the *csoS1* gene of *H. neapolitanus* demonstrated that *Ti*102 and *Ti*108, but not *Ti*104, contained at least parts of both *cbbL* and *csoS1* genes (unpublished). On the basis of preliminary restriction mapping, *SmaI* subclones of pTiCbbL-S and pTicsoS-S were prepared in the vector pT7/T3α18. These subclones, along with *Ti*102, were used for sequencing.

Nucleotide sequence analysis. Automated sequencing of both DNA strands was accomplished with an ABI PRISM Dye Terminator Cycle Sequencing Core Kit, a Perkin-Elmer Cetus DNA thermal cycler, and an ABI 373a DNA sequencer. Universal primers were used for initial reactions, and sequencing oligonucleotide primers were designed for subsequent reactions (Integrated DNA Technologies, Coralville, IA). The nucleotide sequence was analyzed by using GeneWorks software (IntelliGenetics, Mountain View, CA). Amino acid sequences were aligned with ClustalW 1.8 [30]. The percentage identity and percentage similarity values were calculated by using GeneDoc version 2.3 [18]. The nucleotide sequence data reported in this article are available from GenBank under accession numbers AF038430, AF046933, AF129925, and AF307090.

Results and Discussion

The cluster of carboxysome genes in *Tb. denitrificans*, unlike those of *H. neapolitanus* and other thiobacilli, is not closely associated with either *cbbLS* or *cbbM* (Fig. 1). In addition, a *cbbR* gene, which encodes a member of the LysR family of transcriptional regulators [29], re-

Table 1. Percentage amino acid identities of carboxysome peptides of the thiobacilli

CbbL	*Tbd*	*Af*	*Tmi*	*Hn*	CsoS3	*Tbd*	*Af*	*Tmi*	*Hn*	CsoS1C	*Tbd*	*Af*	*Tmi*	*Hn*
	Tbd	86	90	89		*Tbd*	49	38	44		*Tbd*	84	86	88
		Af	86	89			*Af*	40	47			*Af*	85	89
			Tmi	90				*Tmi*	38				*Tmi*	87
				Hn					*Hn*					*Hn*
CbbS	*Tbd*	*Af*	*Tmi*	*Hn*	ORF A	*Tbd*	*Af*	*Tmi*	*Hn*	CsoS1A	*Tbd*	*Af*	*Tmi*	*Hn*
	Tbd	55	49	53		*Tbd*	79	80	75		*Tbd*	84	86	88
		Af	63	60			*Af*	78	74			*Af*	90	91
			Tmi	62				*Tmi*	72				*Tmi*	91
				Hn					*Hn*					*Hn*
CsoS2	*Tbd*	*Af*	*Tmi*	*Hn*	ORF B	*Tbd*	*Af*	*Tmi*	*Hn*	CsoS1B	*Tbd*	*Af*	*Tmi*	*Hn*
	Tbd	39	28	29		*Tbd*	65	61	64		*Tbd*	75	61	68
		Af	29	30			*Af*	53	62			*Af*	58	78
			Tmi	23				*Tmi*	54				*Tmi*	61
				Hn					*Hn*					*Hn*

Tbd = Thiobacillus denitrificans; *Af = Acidithiobacillus ferrooxidans*; *Tmi = Thiomonas intermedia*; *Hn = Halothiobacillus neapolitanus.*

sides just upstream of *csoS2*. This is the third *cbbR* copy identified in *Tb. denitrificans*, in addition to *cbbR* genes upstream from both *cbbLS* and *cbbM* [9, 12]. Interestingly, despite previous and ongoing efforts, carboxysomes have not been detected thus far in this organism [21]. *A. ferrooxidans* also possesses a *cbbR* gene, which is located just upstream from *cbbL*. In *Tm. intermedia*, no *cbbR* gene is present, but a small open reading frame, designated *orfC*, resides immediately upstream of *cbbL* in this organism. It is not clear at this time whether this gene encodes a carboxysome polypeptide. The organization of the downstream carboxysome genes is identical in the four thiobacilli, featuring the gene order: *csoS2*, *csoS3*, *orfA*, *orfB*, *csoS1C*, *csoS1A*, and *csoS1B*.

Primer extension analysis demonstrated a promoter region, residing 54 nucleotides upstream from *cbbL* in *H. neapolitanus* [1, 2], to be:

$$\overset{-35}{\text{ACTGTTATTGATGGCACCGA}}$$

$$\overset{-10}{\text{CTCGGCATGA}}\text{TGCTTTTCA}\overset{+1}{\mathbf{C}}$$

This same region, with a three-nucleotide difference in start site, was predicted with the BDGP:Neural Network Promoter Predictor program (http://www.fruitfly.org/seq_tools/promoter.html). Additional potential promoters were identified with this program in the *orfAB* and *csoS1* regions. Although *cbbS* was not expressed in a *cbbL* insertion mutant and functional carboxysomes were not present, *csoS1* transcripts, albeit at highly reduced levels, were detectable in the mutant by Northern blotting [2]. These results suggest that an additional internal promoter is present in the carboxysome gene cluster. It is unclear at this point whether it directs transcription of the downstream carboxysome genes in the wild type under normal physiological conditions, or whether it is cryptic and used only when the major *cbbL* promoter is nonfunctional. The BDGP:Neural Network Promoter Predictor program detected a putative promoter immediately upstream from *cbbL*, or from *csoS2* for *Tb. denitrificans*, for the carboxysome gene clusters in the three other thiobacilli. A potential promoter was also detected upstream from *orfC* in *Tm. intermedia*. These data suggest the existence of a carboxysome operon in the thiobacilli but require further transcriptional analyses for verification of this assumption.

Although apparently having differing translational capabilities, a classical Shine-Dalgarno ribosome-binding site is found for each carboxysome gene in the cluster, and with the exception of the carboxysome gene cluster in *Tm. intermedia* for which downstream sequence information is not yet available, each putative operon had a stem-loop structure that could function as a transcriptional termination site downstream from *csoS1B* (not shown). Interestingly, a putative termination stem-loop was also noted downstream from *cbbS* in the three thiobacilli whose operon commences with *cbbL*. Since RuBisCO is by far the most abundant protein of the carboxysome, this feature might allow the bacteria to produce this protein in excess while limiting the production of the other carboxysome components via regulated transcriptional termination.

The peptides encoded by the genes of the four carboxysome operons exhibit varying degrees of homology (Table 1). Predictably, the *cbbL* gene products are highly conserved among the thiobacilli, whereas those of *cbbS* show much less identity, as discussed in detail by Tabita [29]. The CsoS1A, CsoS1B, and CsoS1C proteins are encoded by a three-gene repeat in each organism [8, 24]. CsoS1A and CsoS1C are nearly identical (>90%) in each organism and are also highly conserved (84–91%

A. ferrooxidans	249	**VTG**	TQVERSAK	**VTG**	AEPGSCLA	**ITG**	TEYIGTEQYGALCTATPEPAPAKVNVGRTTRGQR
H. neapolitanus	272	**VTG**	TQVDRKSH	**VTG**	NEPGTCRA	**VTG**	TEYVGTEQFTSFCNTSPKPNATKVNVTTTARGRP
Tm. intermedia	291	**VTG**	TQVERSQR	**VSG**	NEPGSCRA	**ITG**	TEYIGSEQFDTLCKTRPAPNPPKVGVSTTLREQR
Tb. denitrificans	143	**VTG**	NQVERTTR	**VTG**	NESGTCRT	**VTG**	TEYVGAEQFGEFCGTLPEPAPAKVGQTSTSRGRR
A. ferrooxidans	308	**VSG**	VELGHSVK	**VTG**	DEHGTCKA	**VTG**	TEYLSADKFESFCATRPALTPAKVSVAATEAGQR
H. neapolitanus	331	**VSG**	TEVSRTEK	**VTG**	NESGVCRN	**VTG**	TEYMSNEAHFSLCGTAAKPSQADKVMFGATARTHQV
Tm. intermedia	350	**VTG**	TEVGRSSK	**MTG**	DEPGSCRV	**ITG**	TDYLSAERYQEFCDTRPQPGAQKVGRGTTEMGQR
Tb. denitrificans	202	**VTG**	TEVGRSTR	**VTG**	DETGTCKR	**VTG**	TEYLAAEQAGEFCGTTPEPRPEKAVMGMTAARNA
A. ferrooxidans	367	**VSG**	TEVGRSAR	**VTG**	DEPGSCRK	**LTG**	SQYYQPESFGSLCRDGGSAPHKVSVMSTLREHA
H. neapolitanus	392	**VSG**	SDEFRPSS	**VTG**	NESGAKRT	**ITG**	SQYADEGLARLTINGAPAKVARTHTFAGSD
Tm. intermedia	409	**FTG**	TLVDRPVK	**VTG**	GEQGSDRT	**VTG**	TSYSMASADTAPNKVEISTTAQGKA
Tb. denitrificans	261	**VSG**	SDLARNVN	**VTG**	GEAGAARS	**ITG**	SAYADGSARSTTEGRGPKKVETRRTGAGAT

Fig. 2. Alignment of the first nine three-amino acid repeats found in the carboxysome shell protein CsoS2. The short repeats consisting of three residues are shown in **bold**. Each set of three short repeats with interspaced amino acids constitutes a large repeat, which is found eight times in the primary structure of CsoS2. The numbers indicate the position of the first residue in the large repeat.

identity) among the four bacteria. With its extended C-terminus, CsoS1B is somewhat larger than the other two homologs and, consequently, displays lower homology values (58–75% identity). If one disregards the C-terminal extension for analysis purposes, the identity values between CsoS1B, CsoS1A, and CsoS1C are greater than 80% within one organism and at least 77% between organisms.

The ORF A and ORF B peptides exhibit 72–80% and 53–65% identities, respectively, between organisms (Table 1). These data, along with the peptide alignments (not shown), support the conclusion that *orfA* and *orfB* represent a two-gene repeat; however, the selective pressure to maintain the sequence of these peptides must not be as great as it is for CsoS1A, CsoS1B, and CsoS1C, since the identity is only 35–48% between ORF A and ORF B sequences of the four organisms. Carboxysomal location and function of ORF A and ORF B in the microcompartment have yet to be determined.

The *csoS2* and *csoS3* gene products are not so well conserved, with identities only ranging from 23% to 39% and from 38% to 49% for CsoS2 and CsoS3, respectively. The identification of CsoS3 as a carboxysomal shell constituent has been reported [4]. CsoS2, which is the least conserved of all carboxysome proteins, possesses some unusual sequence characteristics. Three-amino acid repeats are evident (bold-faced in Fig. 2) that occur more than 20 times in the primary sequence of the protein. The most common residue in the first position of this short repeat is valine (63%), followed by isoleucine (19%), leucine (9%), and methionine (5%). Phenylalanine, threonine, and tyrosine occur as first residues in two or three of the repeats. The second position is occupied by threonine (81%) or serine (19%), and the third residue is invariably a glycine. These three-residue repeats are part of larger repetitive arrangements (Fig. 2) that encompass three short repeats and occur eight times in the primary sequence. The first two short motifs of the larger repeats are separated by eight amino acids, while the distance between the third short motif and the beginning of the next large repeat is 30–40 residues long. The two amino acids immediately following the three-amino acid short repeats also show some, albeit less pronounced, regularity. Acidic and uncharged polar residues preferentially occupy the two positions immediately following the glycine of the short repeat. Threonine (39%), aspartate (18%), asparagine (14%), and serine (11%) tend to be present in the first position after the short repeats, whereas glutamate (42%) and glutamine (12%) most often occupy the subsequent position. The implications of these unique structural features for the function of CsoS2 as a carboxysomal shell protein and for its interactions with other components of the prokaryotic microcompartment are currently unknown and underscore the need for additional research efforts that will further our understanding of the ways in which the structure of the carboxysomal protein components affects the function of the microcompartment.

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