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ORIGINAL PAPER

Cnb proteins affect the intracellular 2-MIB retention in heterologous gene expression systems

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Abstract
2-methylisoborneol (2-MIB) is one of the main cyanobacterial metabolites responsible for unpleasant taste and odor in drinking water. 2-MIB biosynthesis involves a two-step enzymatic reaction catalyzed by enzymes encoded by the *mtf* and *mtc* genes. Two homologous genes, *cnb*A and *cnb*B, which encode cyclic nucleotide-binding proteins of unknown function are located upstream and downstream of the *mtf* and *mtc* genes, respectively. This study investigated the involvement of *cnb* genes of 2-MIB-producing cyanobacterium *Pseudanabaena foetida* var. *intermedia* NIES 512 in 2-MIB synthesis using heterologous gene expression systems. Transgenic *Escherichia coli* and *Synechococcus elongatus* PCC 7942 strains expressing the *cnb*A-*mtf*-*mtc*-*cnb*B gene cluster exhibited a notable intracellular 2-MIB retention which was not shown by the transgenic strains expressing only the *mtf*-*mtc* gene unit. Immunoelectron microscopy detected that the Cnb proteins are associated with unknown intracellular compartments in transgenic *S. elongatus* PCC 7942 cells. This study suggests that 2-MIB-related Cnb proteins are involved in the formation of intracellular compartments, which are likely associated with intracellular 2-MIB retention.

Keywords Cyanobacteria · Cnb proteins · Intracellular compartments · 2-MIB retention

Introduction

Microbial terpenoids are a diverse group of secondary metabolites synthesized by microorganisms, playing crucial roles in various physiological processes, including signaling, defense mechanisms, and ecological interactions (Avalos et al. 2021). Among these compounds, 2-methylisoborneol (2-MIB) and geosmin are prominent odorous terpenoids that degrade the quality of drinking water and freshwater fish due to their characteristic musty odor and taste. Cyanobacteria and actinomycetes are major producers of 2-MIB (Giglio et al. 2011). However, the biological role of 2-MIB in these organisms is not yet identified. Biosynthesis of 2-MIB involves two enzymatic reactions: the S-adenosyl methionine (SAM) dependent methylation of geranyl diphosphate (GPP) to 2-methyl GPP by geranyl diphosphate methyl transferase (GPPMT) and cyclization of 2-methyl GPP to 2-MIB by monoterpene cyclase (MIBS) (Dickschat et al. 2007). GPPMT and MIBS are encoded by adjacent *mtf* and *mtc* genes respectively. These two enzymatic genes are flanked by the *cnb* genes that encode cyclic nucleotide-binding proteins in cyanobacteria and actinomycetes (Wang et al. 2011). Given the conserved nature of these *cnb* genes with enzymatic genes involved in 2-MIB biosynthesis across cyanobacterial and actinomycetes genomes, they are thought to play a role in 2-MIB production. However, the

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specific function of 2-MIB-related cyclic nucleotide-binding proteins remains unexplored.

Cyclic nucleotide-binding proteins are characterized by the presence of a cyclic nucleotide-binding domain and their ability to bind cyclic nucleotides. Notable examples of cyclic nucleotide-binding proteins include catabolic activator proteins (CAP), cyclic AMP (cAMP) and cyclic GMP (cGMP) kinases, cyclic nucleotide-gated ion channels, and phosphodiesterases (PDEs), which play crucial roles in regulating gene transcription and cellular signaling in both eukaryotes and prokaryotes. (Kannan et al. 2009; Shabb and Corbin 1992). Encapsulins are a newly identified class of prokaryotic protein compartments capable of carrying cargo enzymes within a shell-like structure (Andreas and Giessen 2021; Nichols et al. 2017). Among the currently known four encapsulin families, family two encapsulins have further been divided into two subgroups based on the presence of a cyclic nucleotide-binding domain. Subfamily 2 A encapsulins lack a cyclic nucleotide-binding domain, whereas subfamily 2B encapsulins possess this domain (Giessen 2022; Nichols et al. 2021).

Pseudanabaena foetida var. *intermedia* is a 2-MIB-producing filamentous freshwater cyanobacterium (Tuji and Niiyama 2016). Our previous study showed that the 2-MIB-related gene cluster in *P. foetida* var. *intermedia* NIES 512 consists of the co-expressed *mtf* and *mtc* genes and independently expressed *cnb*A and *cnb*B genes located upstream and downstream of *mtf* and *mtc* genes. That study clarified the heterologous expression of *mtf* and *mtc* genes of *P. foetida* var. *intermedia* NIES 512 is sufficient to synthesize 2-MIB in *Escherichia coli* (Dayarathne et al. 2023). In the present study, a new heterologous gene expression system was constructed using *Synechococcus elongatus* PCC 7942 in addition to the *E.coli* system for 2-MIB-related gene expression. The objective of this study was to investigate the function of *cnb* genes of *P. foetida* var. *intermedia* NIES 512 in the heterologous gene expression systems which will be important in understanding the cellular mechanisms related to 2-MIB biosynthesis.

Materials and methods

Cyanobacteria strains and culture conditions

A 2-MIB-producing cyanobacterium, *P. foetida* var. *intermedia* NIES 512, obtained from the microbial culture collection at the National Institute for Environmental Studies (NIES, Japan), and a non-2-MIB producing cyanobacterium, *S. elongatus* PCC 7942, kindly provided by Prof. Yukako Hihara (Saitama University, Japan) were used in this study. Both species were cultured in liquid BG-11 medium (pH 8.0) (Rippka et al. 1979) under a light intensity of 20 µmol photons/m^2/s. *P. foetida* var. *intermedia* NIES 512 was incubated at 22 °C under static conditions, whereas *S. elongatus* PCC 7942 was maintained at 30 °C with continuous shaking at 80 rpm.

Bioinformatic analysis of cnb proteins of *P. foetida* var. *Intermedia*

The amino acid sequences of cyclic nucleotide-binding protein A (CnbA) and cyclic nucleotide-binding protein B (CnbB) of *P. foetida* var. *intermedia* NIES 512 were analyzed using the GenomeNet motif search tool (https://www.genome.jp › tools › motif) to detect specific protein motifs in Cnb proteins. The molecular weights of CnbA and CnbB proteins were estimated by the ExPASy ProtParam tool (https://web.expasy.org/protparam/). Homologous amino acid sequences of the CnbA and CnbB proteins of *P. foetida* var. *intermedia* NIES 512 were identified using the BLASTP algorithm, NCBI (www.blast.ncbi.nlm.nih.gov/Blast.cgi) against the bacterial protein sequence database. Retrieved amino acid sequences were further investigated for specific protein motifs.

Construction of transgenic *E. coli* and *S. elongatus* PCC 7942 strains

Gene fragments *cnb*A-*mtf-mtc*, *mtf-mtc-cnb*B, and *cnb*A-*mtf-mtc-cnb*B were amplified from the genomic DNA of *P. foetida* var. *intermedia* NIES 512 using the primers mentioned in Dayarathne et al. 2023. Each gene fragment was separately inserted into the expression vector pYS1C carrying chloramphenicol-resistant gene designed by Sakamaki et al. (2022). In-Fusion HD cloning Kit (Takara Bio, Japan) was used for the cloning, following the manufacturer's protocol. The resulting plasmid constructs (pYS1C-cnbA-mtf-mtc, pYS1C-mtf-mtc-cnbB, and pYS1C-cnbA-mtf-mtc-cnbB) were verified by DNA sequencing and transformed into *E. coli* JM109 cells using the heat shock method as described by Dagert and Ehrlich (1979), with modifications. For transformation, an overnight culture of *E. coli* JM109 was diluted 1:100 into fresh LB medium (Bertani 1951) and incubated for 3 h. Cells were harvested by centrifugation at 3000 rpm for 5 min and induced for competency by resuspending the pellet in ice-cold 50 mM $CaCl_2$ in 10 mM Tris-HCl (pH 8.0). After incubation on ice for 30 min, plasmid DNA (0.01–1 µg) from each plasmid construct was separately added to 200 µl of competent cells, and the mixture was incubated on ice for an additional 30 min. Heat shock was applied at 41 °C for one min, after which the cells were immediately cooled on ice and allowed to recover in LB medium at 37 °C for 1 h. Transformed cells were plated on

LB agar supplemented with chloramphenicol (35 µg/ml) and incubated overnight at 37 °C. Successful transformants carrying respective plasmids were confirmed by colony PCR.

To construct a new cyanobacterial heterologous gene expression system, the pYS1C vector containing the *mtf* and *mtc* genes from *P. foetida var. intermedia* NIES 512 described in Dayarathne et al. 2023 was introduced into wild-type *S. elongatus* PCC 7942. Following successful confirmation of 2-MIB synthesis, other plasmid vectors (pYS1C-cnbA-mtf-mtc, pYS1C-mtf-mtc-cnbB, and pYS1C-cnbA-mtf-mtc-cnbB) were also introduced to wild-type *S. elongatus* PCC 7942 cells independently. Plasmids were introduced into cyanobacterial cells via natural transformation, as described below. Cells were grown to an OD_{730} of 0.8–1, and harvested by centrifuging 10 ml of culture at 8000 rpm for 5 min, followed by resuspension in 1 ml of BG-11 medium. To 200 µl of cell suspension, 0.01–1 µg of plasmid DNA was added, and the mixtures were incubated overnight at 30 °C with gentle rotation in the dark. After the completion of overnight incubation, cells were exposed to light at 20 µmol photons/m²/s for 1 h. The transformation mixtures were plated on BG-11 agar containing 7.5 µg/ml chloramphenicol and incubated at 30 °C under 20 µmol photons/m²/s of light intensity for 2–3 weeks to allow colony formation. After the confirmation of successful transformants by genomic PCR, transgenic cultures were maintained in liquid BG-11 medium supplemented with 7.5 µg/ml chloramphenicol under the same conditions used for the wild-type *S. elongatus* PCC 7942 culture.

2-MIB analysis by gas chromatography-mass spectrometry (GC-MS)

For the analysis of 2-MIB production in transgenic *E. coli* strains, overnight cultures were diluted 1:100 into fresh LB medium containing 35 µg/ml chloramphenicol and cultured at 37 °C under constant shaking at 140 rpm. Gene expression was facilitated by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the medium 2 h after the inoculation. Cells were harvested for the GC-MS analysis after 5 h of the inoculation. 2-MIB production in wild-type *P. foetida* var. *intermedia* NIES 512 and transgenic *S. elongatus* PCC 7942 strains were analyzed using the cells collected at mid-exponential phase (12–15 days-old cultures). To induce the gene expression in transgenic *S. elongatus* PCC 7942 strains, 1 mM IPTG was added to the cultures 5 days after the inoculation. 2-MIB production was quantified separately in the intracellular, extracellular, and total fractions of each cell sample. The optical density of each *E. coli* and cyanobacterial sample was measured at 600 nm (OD_{600}) and 730 nm (OD_{730}) respectively, using a spectrophotometer (Ultrospec 3000; Pharmacia Biotech, Midland, Canada) before the 2-MIB analysis.

Extracellular 2-MIB was quantified using the culture supernatant obtained by centrifuging 1 ml of culture at 8000 g for 5 min at 25 °C. Intracellular 2-MIB was measured by resuspending the resulting cell pellet in 1 ml of distilled water. For the analysis of total 2-MIB content, untreated culture samples were used without prior separation. Hexane extraction of 2-MIB was performed by mixing the test samples, methanol, and hexane in 1:1:1 ratio followed by vigorous vertexing and subsequent centrifugation at 120 *g* for 10 min at 25 °C. The hexane layer was taken for the quantification of 2-MIB by GC-MS (Shimadzu GCMS-QP2010) equipped with a TC-70 column (0.25 µm × 0.25 mm × 30 m, GL Science, Japan) using He as the carrier gas at 37.7 cm/s of linear velocity and the temperature program; held at 60 °C for 3 min, 20 °C/min to 200 °C, held for 5 min. Total ion scanning or selected ion monitoring (SIM) mode was used to detect 2-MIB. The 2-MIB concentration was determined using a standard curve constructed with the known concentrations of the 2-MIB standard (FUJIFILM Wako Chemicals, USA). The values were normalized by dividing by the OD value of the respective culture sample and presented as concentration per biomass as described in Dayarathne et al. 2024. The percentages of intracellular and extracellular 2-MIB levels of each transgenic strain were calculated relative to the total 2-MIB levels produced by each strain.

Growth analysis of transgenic *E. coli* and *S. elongatus* PCC 7942 strains

For the growth analysis, both transgenic *E. coli* and *S. elongatus* PCC 7942 strains were cultured under IPTG induction following the same steps mentioned in the previous section. The growth of *E. coli* was monitored by measuring the OD_{600} of cultures hourly for 10 h whereas the growth of *S. elongatus* PCC 7942 strains was assessed by measuring OD_{730} of cultures at 3-day intervals over 15 days.

Antibody preparation and validation

Polyclonal rabbit antibody was ordered from Eurofin Genomics (Japan) for the immunodetection of CnbA and CnbB proteins. The antibody was produced targeting a short peptide sequence (ELRERQEYELVNNREFG), conserved in the highly homologous CnbA and CnbB proteins, and purified by affinity chromatography. Western blot analysis was performed to confirm the specificity of the antibody prior to use in the immune-electron microscopy. Total protein lysates (15 µg) from wild-type *P. foetida* var. *intermedia* NIES 512, transgenic *S. elongatus* PCC 7942-pYS1C-mtf-mtc, and

transgenic *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB cells were separated by 12% SDS-PAGE. The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane and probed with the Cnb antibody at a dilution of 1:1000. Horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, United Kingdom) at a dilution of 1:2000 and enhanced chemiluminescence (ECL) detection system (GE Healthcare, Japan) were used for the visualization of the protein bands under chemiluminescence imager (Bio-Rad, USA).

Electron microscopic observations

Wild-type cells of *P. foetida* var. *intermedia* NIES 512 and *S. elongatus* PCC 7942, along with transgenic *S. elongatus* PCC 7942 strains (*S. elongatus* PCC 7942-pYS1C-mtf-mtc, and *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB) were observed under transmission electron microscope (TEM). Cyanobacteria cells were collected from 12 days-old cultures. Cells were chemically fixed using 2% glutaraldehyde and 2% osmium tetroxide, followed by dehydration with washing steps with 10–100% acetone. After embedding the dehydrated cells into spurr resin, ultrathin sections with a thickness of 90 nm were cut using an ultramicrotome (UltracutN, Nissei, Japan), and the sections were stained with uranyl acetate for 10 min, followed by staining with lead citrate for 1 min. Sections were observed under a transmission electron microscope Hitachi H-7500 (Hitachi, Tokyo, Japan). Immunoelectron microscopy was performed by immunostaining of ultrathin sections of cyanobacterial samples (Wild-type cells of *P. foetida* var. *intermedia* NIES 512, *S. elongatus* PCC 7942-pYS1C-mtf-mtc, and *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB) with Cnb antibody. Secondary antibodies conjugated to 10 nm colloidal gold particles were used for the detection following the method described by Atsuzawa et al. (2010) with modifications.

Results and discussion

Structural analysis and comparison of cnb proteins

The predicted molecular weights of CnbA and CnbB proteins of *P. foetida* var. *intermedia* NIES 512 were 52.05 and 52.12 kDa, respectively. CLUSTALW pairwise sequence alignment revealed 81% amino acid sequence identity between CnbA and CnbB. Both CnbA and CnbB proteins were found to consist of two domains: type 2B encapsulin shell protein SrpI-like domain (252 AA) and cyclic nucleotide-binding domain (87 AA). The encapsulin proteins are known to form nanocompartments that can encapsulate specific enzymes or metabolites (Giessen 2022). A previous study reported a prokaryotic nanocompartment protein family, which is commonly found as a two-gene system, with one gene encoding a shell protein that forms an icosahedral capsid-like structure and the other gene encoding a cargo protein that is encapsulated within the shell (Nichols et al. 2021). Their study further described that family 2B nanocompartment protein-encoding genes are found adjacent to the terpenoid synthesis genes with CRP/FNR cyclic nucleotide-binding domain. Therefore, the *cnb* genes found in the 2-MIB-related gene cluster of *P. foetida* var. *intermedia* NIES 512 can be suspected as family 2B nanocompartment protein-encoding genes. Generally, cyclic nucleotide-binding domains are known to be involved in various cellular processes by binding cyclic nucleotides (Kornev et al. 2019). The binding of cyclic nucleotides to the cyclic nucleotide-binding domains of different proteins induces conformational changes that modulate their activity. This mechanism has been described in various proteins in previous studies (Zhou et al. 2012; Zagotta et al. 2003; Busby and Ebright 1999). Given these examples, it is reasonable to suggest that the binding of cyclic nucleotides to the cyclic nucleotide-binding domains of 2-MIB-related CnbA and CnbB proteins could similarly induce conformational changes facilitating the formation of nanocomparment-like structures.

Bioinformatics analysis further demonstrated that cyclic nucleotide-binding proteins from other 2-MIB-producing cyanobacteria (*Pseudanabaena* sp. dqh15, *Planktothricoides* sp., *Planktothricoides raciborskii*), actinomycetes (*Streptomyces lasalocidi*, *Streptomyces ambofaciens*, *Streptomyces griseus*), and geosmin-producing cyanobacteria (*Calothrix* sp., *Cylindrospermum stagnale*, *Nostoc punctiforme*) also commonly possess the cyclic nucleotide-binding and the SrpI-like domains as conserved structural features (Fig. 1). The CnbA and CnbB proteins of *P. foetida* var. *intermedia* NIES 512 showed high sequence identity with those of other 2-MIB-producing cyanobacteria. Especially, *P. foetida* var. *intermedia* NIES 512 Cnb proteins share 99–100% identity, with Cnb proteins of *Pseudanabaena* sp. dqh15. Additionally, the Cnb proteins of *P. foetida* var. *intermedia* NIES 512 display substantial identity (approximately 50–60%) with Cnb proteins from 2-MIB-producing actinomycetes and geosmin-producing cyanobacteria. These conserved structural domains and high sequence similarities among Cnb proteins from 2-MIB or geosmin-producing cyanobacteria and actinomycetes suggest that Cnb proteins may share similar functions in the 2-MIB or geosmin biosynthesis process.

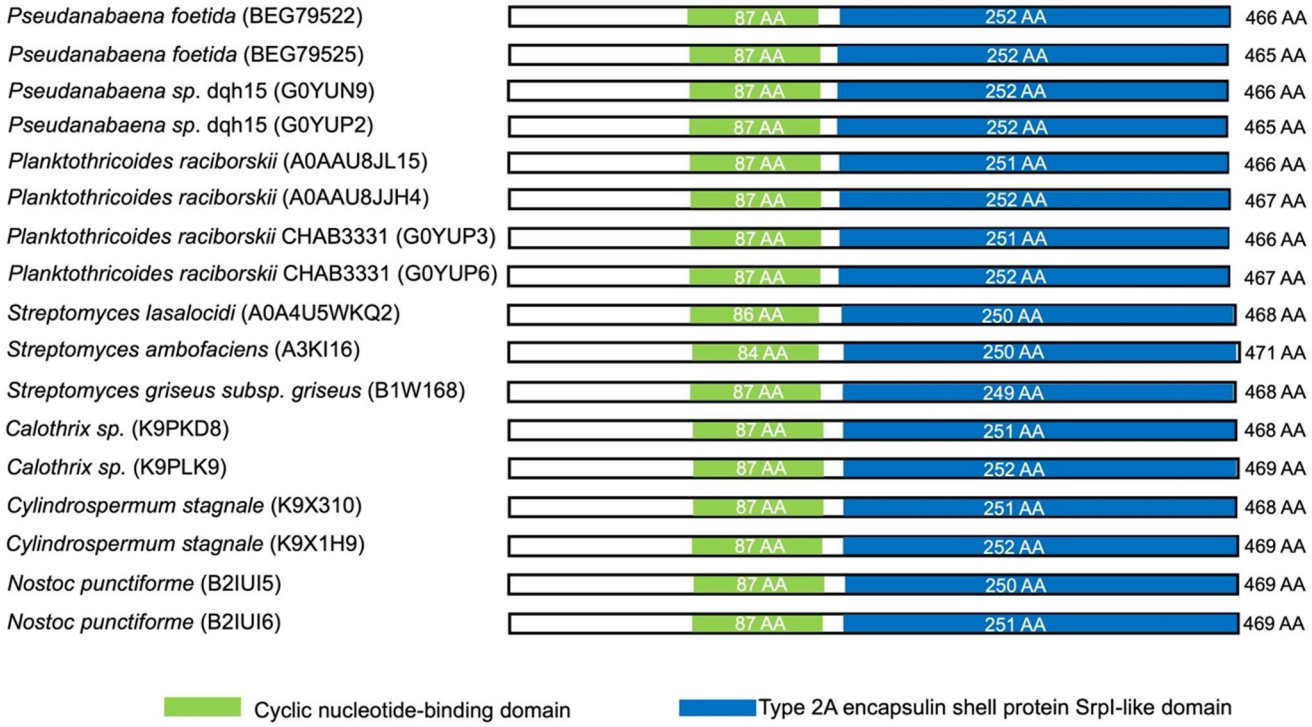

Fig. 1 Structural comparison of 2-MIB and geosmin-related Cnb proteins showing conserved cyclic nucleotide-binding and SrpI-like domains. GenBank accession numbers for Cnb proteins of *P. foetida* var. *intermedia* NIES 512 and UniProt accession numbers for Cnb proteins of other strains are indicated in parentheses

The effect of *cnb*A and *cnb*B genes on intracellular 2-MIB retention

Given that the transgenic *E.coli* strain harboring only the *mtf-mtc* gene unit was unable to retain intracellular 2-MIB, unlike the wild-type *P. foetida* var. *intermedia* NIES 512, which showed significant intracellular 2-MIB retention in our previous study (Dayarathne et al. 2023), the present study focused on investigating whether Cnb proteins contribute to intracellular 2-MIB retention. For that, *cnb* genes of *P. foetida* var. *intermedia* NIES 512 were first introduced into *E. coli* along with the *mtf-mtc* gene unit. Among the three transgenic *E. coli* strains tested, the strain, harboring all four 2-MIB-related genes of *P. foetida* var. *intermedia* NIES 512 (*E. coli*-pYS1C-cnbA-mtf-mtc-cnbB), exhibited a higher intracellular 2-MIB concentration (0.004 µg/ml/OD_{600}) compared to the extracellular concentration (0.0018 µg/ml/OD_{600}) (Fig. 2A).

To observe the difference between the two 2-MIB fractions further, the percentages of intracellular and extracellular 2-MIB levels were calculated relative to the total 2-MIB level. In *E. coli*-pYS1C-cnbA-mtf-mtc-cnbB strain, the intracellular 2-MIB percentage (62%) was significantly higher compared to the control strain (*E. coli*-pYS1C-mtf-mtc), which showed only 9% of intracellular 2-MIB retention. Conversely, the extracellular 2-MIB percentage (28%) was significantly lower in the *E. coli*-pYS1C-cnbA-mtf-mtc-cnbB strain (Fig. 2B). Furthermore, *S. elongatus* PCC 7942 was used as a new cyanobacterial heterologous system for 2-MIB-related gene expression in this study. 2-MIB production was achieved by introducing *mtf-mtc* gene unit into the wild-type *S. elongatus* PCC 7942 strain, with the produced 2-MIB predominantly detected in the extracellular fraction. Therefore, any potential role of Cnb proteins in 2-MIB retention in transgenic *S. elongatus* PCC 7942 was investigated by introducing *cnb* genes. However, the intracellular 2-MIB concentrations were significantly lower than the extracellular 2-MIB concentrations in all tested transgenic *S. elongatus* PCC 7942 strains harboring *cnb* genes (Fig. 3A). In comparison with the control strain (*S. elongatus* PCC 7942-pYS1C-mtf-mtc), the intracellular 2-MIB percentages were higher in the transgenic *S. elongatus* PCC 7942 strain harboring all four genes (*S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB) and slightly higher in those with only *cnb*A, *mtf*, and *mtc* genes (*S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc) (Fig. 3B). Although the transgenic *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB strain showed considerable 2-MIB retention compared to the control strain, that 2-MIB retention was significantly lower than that of the transgenic *E. coli*-pYS1C-cnbA-mtf-mtc-cnbB strain. This lower retention might be due to the reduced expression levels of the *cnb* genes in the transgenic

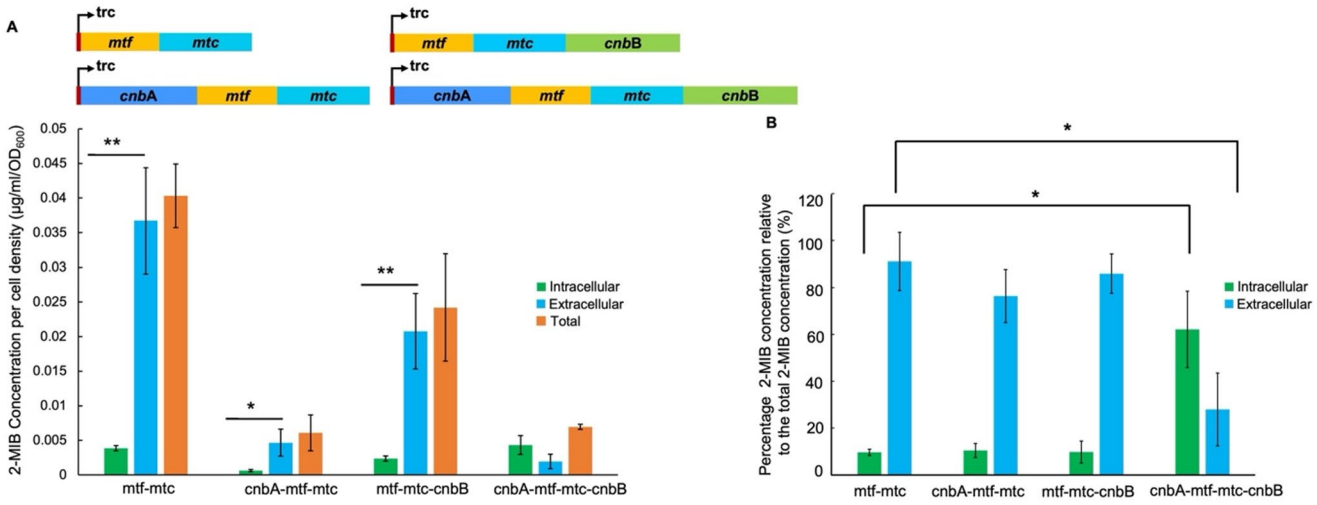

Fig. 2 Intracellular, extracellular, and total 2-MIB levels in transgenic *E.coli* strains: *E.coli*-pYS1C-mtf-mtc, cnbA-mtf-mtc, mtf-mtc-cnbB, and cnbA-mtf-mtc-cnbB as concentration per cell density (**A**) and as percentages compared to the total 2-MIB concentrations (**B**). 2-MIB levels denoted by * and ** are significantly different at $p<0.05$ and $p<0.01$, respectively (t-Test). $n=3$ (mean ± SD)

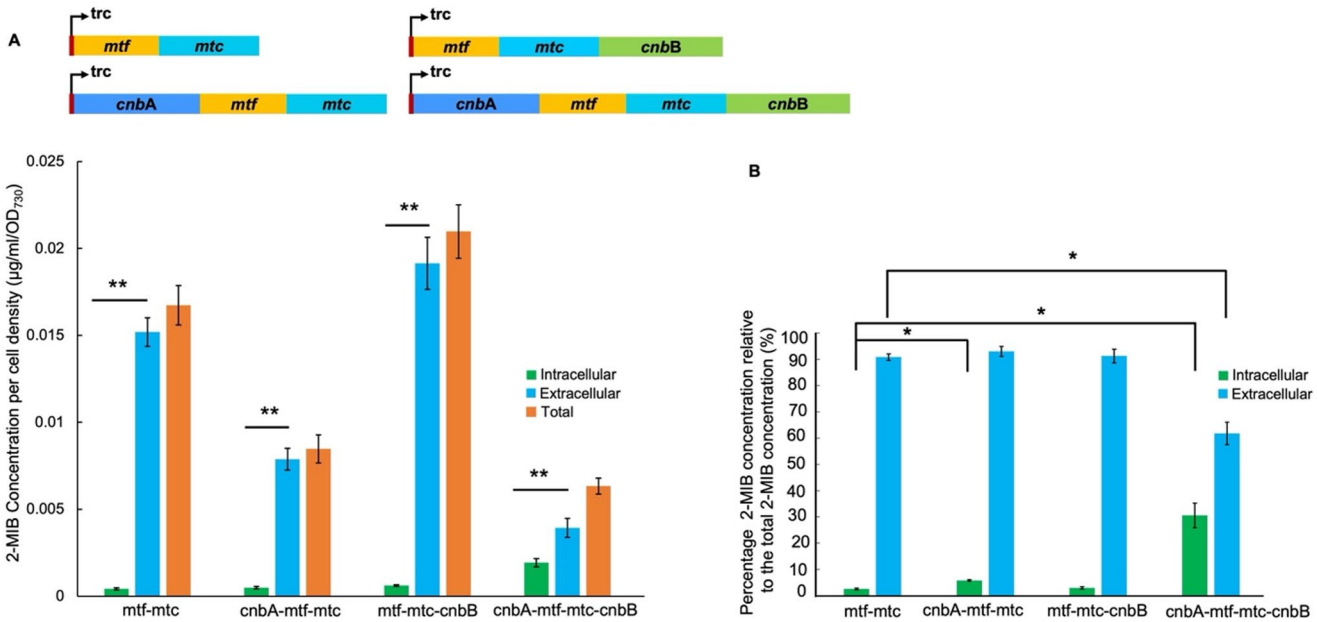

Fig. 3 Intracellular, extracellular and total 2-MIB concentrations in transgenic *S. elongatus* PCC 7942 strains: pYS1C-mtf-mtc, cnbA-mtf-mtc, mtf-mtc-cnbB, and cnbA-mtf-mtc-cnbB as concentration per cell density (**A**) and as percentages compared to the total 2-MIB concentrations (**B**). 2-MIB levels denoted by * and ** are significantly different at $p<0.05$ and $p<0.01$, respectively (t-Test). $n=3$ (mean ± SD)

S. elongatus PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB strain, which could be associated with low activity of *trc* promoter in *S. elongatus* PCC 7942 cells. A similar trend of reduced gene expression level in transgenic *S. elongatus* PCC 7942, compared to the transgenic *E. coli* was reported by Zhou et al. (2014), suggesting lower gene expression strength of *trc* promotor in cyanobacteria than *E. coli*. This limitation was further supported by the findings of the study by Wang et al. (2016), which reported poor expression of a heterologous limonene synthase gene under the *trc* promoter in *S. elongatus* PCC 7942. Their study further demonstrated that both promoter activity and translation efficiency are major limiting factors for heterologous gene expression in cyanobacterial systems. However, intracellular 2-MIB accumulations shown by 2-MIB-producing transgenic *E. coli* and *S. elongatus* PCC 7942 strains harboring both *cnb*A and *cnb*B genes suggest a possible contribution of both *cnb* genes for intracellular 2-MIB retention. The reduced 2-MIB production observed in transgenic *E. coli* and *S. elongatus* PCC 7942 strains harboring pYS1C-cnbA-mtf-mtc and

pYS1C-cnbA-mtf-mtc-cnbB, compared to strains harboring pYS1C-mtf-mtc and pYS1C-mtf-mtc-cnbB might be likely due reduced transcription of *mtf-mtc* gene unit, which could result from transcriptional interference between *trc* promoter and a native promoter for the *mtf-mtc* gene unit located within the *cnb*A gene.

Effect of 2-MIB production on the growth of transgenic strains

The growth of the 2-MIB synthesizing transgenic *E. coli* strains and *S. elongatus* PCC 7942 strains harboring *cnb* genes were compared to the growth of transgenic *E. coli* and *S. elongatus* PCC 7942 strains harboring only *mtf-mtc* gene unit to observe if introduction of *cnb* genes cause any changes in the cell growth. However, no significant difference was observed in the growth of any transgenic *E. coli* (Fig. 4A) or *S. elongatus* PCC 7942 (Fig. 4B) strains harboring *cnb* genes compared to the control strains. These results indicate that retention of 2-MIB has no toxic effects on transgenic *E. coli* and *S. elongatus* PCC 7942 strains.

Intracellular structures associated with 2-MIB synthesizing transgenic *S. elongatus* PCC 7942 strains

To investigate if any special intracellular structures are associated with 2-MIB synthesis and retention in transgenic *S. elongatus* PCC 7942 strains, *S. elongatus* PCC 7942-pYS1C-mtf-mtc and *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB were observed compared to wild-type *S. elongatus* PCC 7942 cells (control) under TEM. There was no notable difference observed in the intracellular morphology between the wild-type *S. elongatus* PCC 7942 cells (Fig. 5A) and the transgenic *S. elongatus* PCC 7942-pYS1C-mtf-mtc cells (Fig. 5B). Unknown intracellular structures with a distinct morphology with approximately 15–20 nm in diameter were observed in transgenic *S. elongatus* 7942-pYS1C-cnbA-mtf-mtc-cnbB (Fig. 5C). A recent study by Andreas and Giessen (2024) described a family 2B encapsulin in the soil bacterium *S. griseus* characterized as a protein nanocompartment with a unique shell architecture and a diameter of 29.4 nm, which facilitates 2-MIB biosynthesis. Notably, the size of the unknown intracellular structures observed in *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB cells is comparable to the nanocompartments reported in that study suggesting a possible functional similarity.

To detect the localization of Cnb proteins within transgenic *S. elongatus* 7942-pYS1C-cnbA-mtf-mtc-cnbB cells and to test if the unknown intracellular structures are composed of Cnb proteins, the cells were subjected to immunoelectron microscopy using Cnb antibody which designed to detect short peptide sequence conserved in CnbA and CnbB proteins. Wild-type *P. foetida* var. *intermedia* NIES 512 and transgenic *S. elongatus* PCC 7942-pYS1C-mtf-mtc cells were also observed as the positive and negative control specimens respectively. The specificity of Cnb antibodies was validated by Western blot analysis prior to immunoelectron microscopy. Clear protein bands were observed at the expected molecular weight of approximately 52 kDa in total protein extracts of both wild-type *P. foetida* var. *intermedia* NIES 512 and transgenic *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB, consistent with the predicted size of the Cnb proteins. No signal was observed in the control strain, transgenic *S. elongatus* PCC 7942-pYS1C-mtf-mtc confirming the specificity of the antibody (Online Resource 1). In immunoelectron microscopy, cytoplasmic localization of Cnb proteins was observed in wild-type *P. foetida* var. *intermedia* NIES 512 (Fig. 6A). The immunosignals for Cnb proteins were not detected in the cells of the negative control, *S. elongatus* PCC 7942-pYS1C-mtf-mtc (Fig. 6B) confirming the specificity of Cnb antibodies. In the transgenic *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB strain, Cnb proteins were detected in the vicinity of unknown intracellular structures (Fig. 6C) suggesting a potential role of 2-MIB-related Cnb proteins in forming special intracellular compartments. However, the immunosignals for Cnb proteins detected in the cytoplasm of wild-type *P. foetida* var. *intermedia* NIES 512 cells were not associated with aggregations of distinct intracellular structures as observed in the transgenic *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB strain. One possible reason for that difference is that Cnb protein-related intracellular compartments in the wild-type *P. foetida* var. *intermedia* NIES 512 cells might be relatively smaller and randomly distributed in the cytoplasm. Moreover, these compartments may be smaller than the 10 nm gold particles used for detection, which could make them difficult to visualize as distinct structures. We hypothesize that small unknown intracellular structures observed in transgenic *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB cells might be specialized intracellular compartments made of Cnb proteins which could be involved in intracellular 2-MIB retention.

Conclusion

P. foetida var. *intermedia* NIES 512 CnbA and CnbB proteins possess type 2B encapsulin shell protein SrpI-like domain and cyclic nucleotide-binding domain. Heterologous expression of CnbA and CnbB proteins demonstrated their roles related to intracellular 2-MIB accumulation in transgenic *E. coli* and *S. elongatus* 7942 strains. We hypothesize that

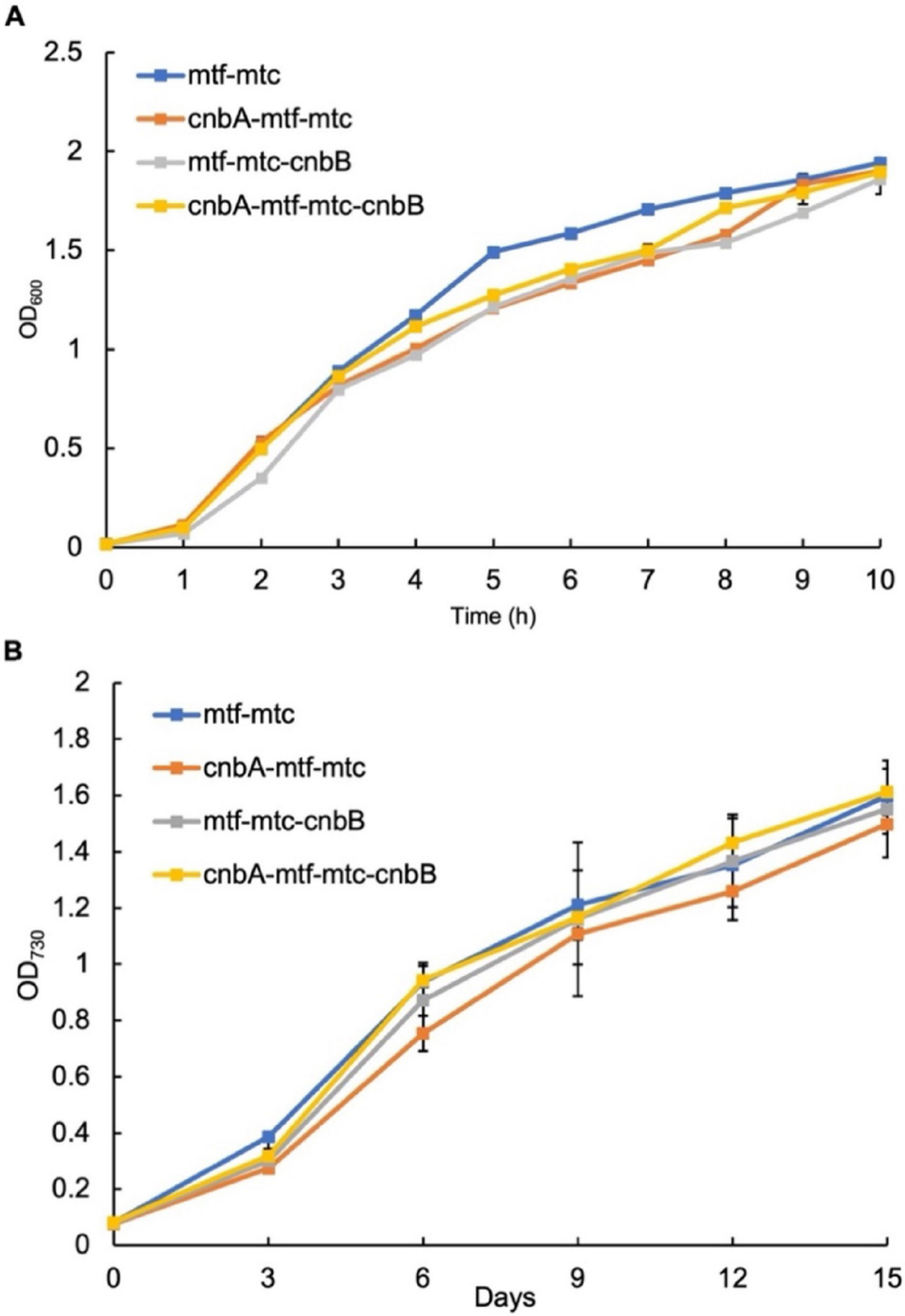
A
mtf-mtc
cnbA-mtf-mtc
mtf-mtc-cnbB
cnbA-mtf-mtc-cnbB
OD600
Time (h)
B
mtf-mtc
cnbA-mtf-mtc
mtf-mtc-cnbB
cnbA-mtf-mtc-cnbB
OD730
Days

Fig. 4 The growth of transgenic *E. coli* strains: pYS1C-mtf-mtc, cnbA-mtf-mtc, mtf-mtc-cnbB, and cnbA-mtf-mtc-cnbB (**A**) and transgenic *S. elongatus* PCC 7942 strains: pYS1C-mtf-mtc, cnbA-mtf-mtc, mtf-mtc-cnbB, and cnbA-mtf-mtc-cnbB (**B**). $n = 3$ (mean ± SD)

the Cnb proteins of transgenic *S. elongatus* 7942 are likely involved in intracellular compartmentalization providing a vicinity for intracellular 2-MIB retention. Since distinct intracellular compartments were not observed in wild-type *P. foetida* var. *intermedia* NIES-512 cells, we hypothesize that the assembly, stability, and size of Cnb-related intracellular compartments vary across species. Therefore, further investigations such as immunoprecipitation of Cnb proteins followed by cryo-electron microscopic observations are required to observe the Cnb-related intracellular compartments in wild-type *P. foetida* var. *intermedia* NIES 512 cells and transgenic *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB. Furthermore, the construction of *cnb* knockout strains of *P. foetida* var. *intermedia* NIES 512 is necessary to determine the precise involvement of the *cnb*A and *cnb*B genes in 2-MIB biosynthesis.

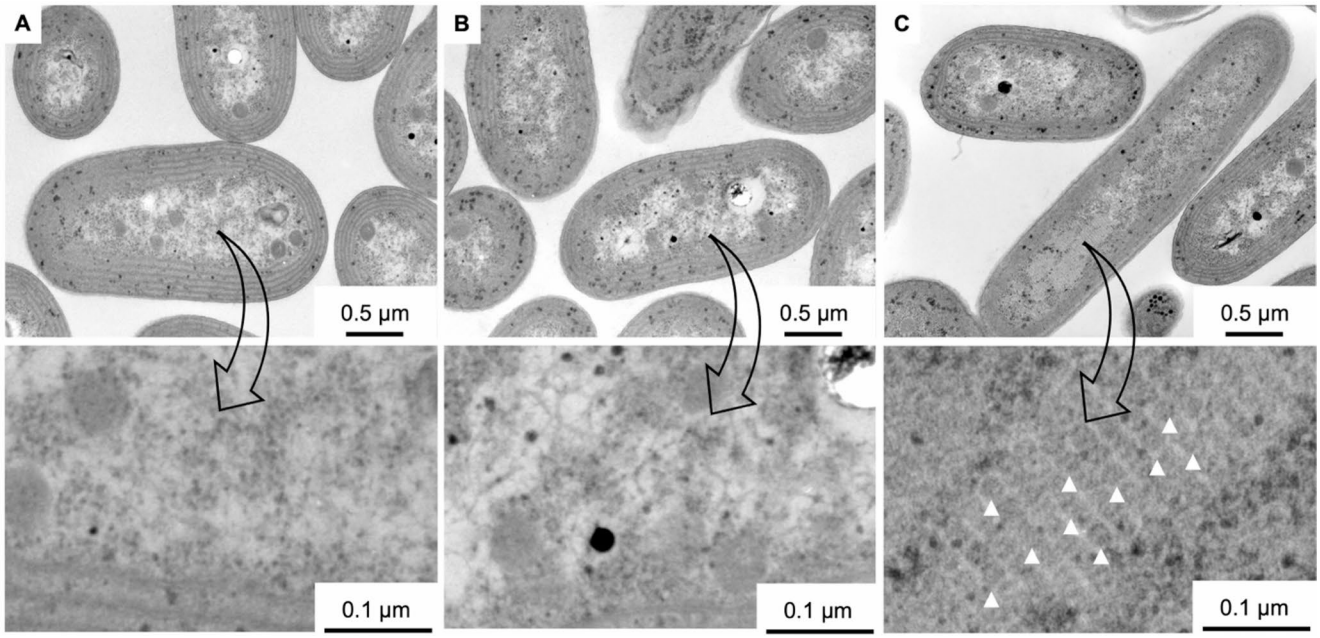

Fig. 5 TEM images of wild-type *S. elongatus* PCC 7942 (**A**), transgenic *S. elongatus* PCC 7942-pYS1C-mtf-mtc (**B**) and transgenic *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB (**C**). In the magnified view of transgenic *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB cell, white color arrow heads indicate closely assembled unknown intracellular structures with a distinct morphology which were not observed in the other two strains

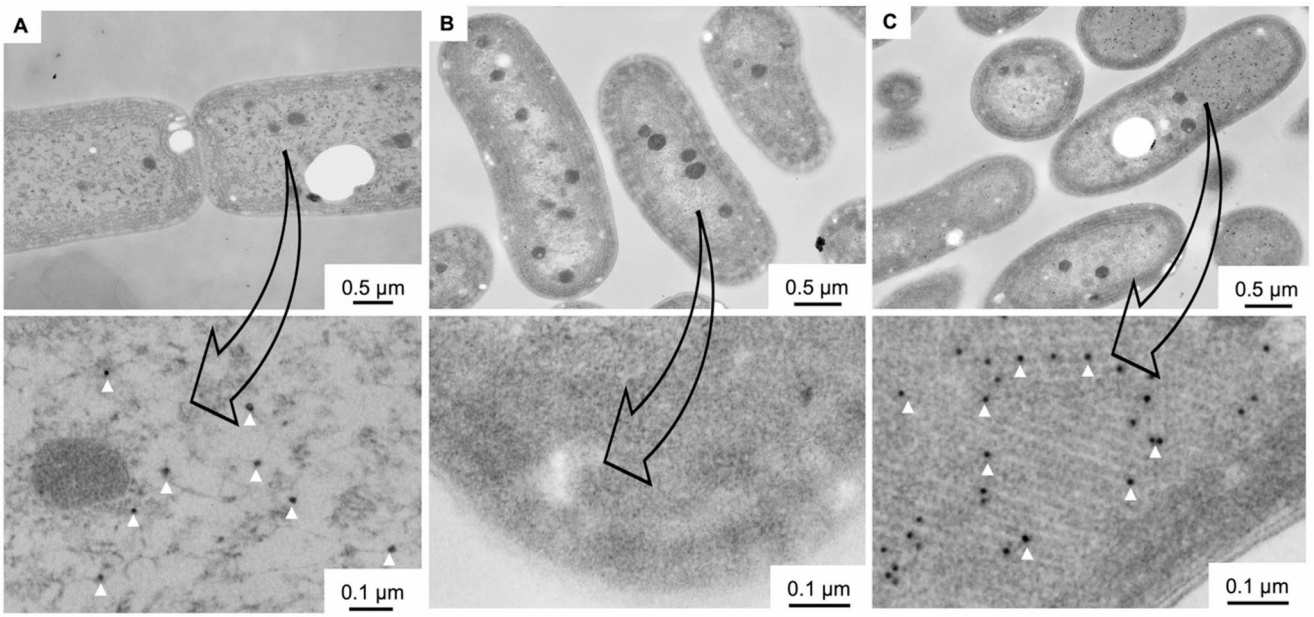

Fig. 6 Immunoelectron micrographs of wild-type *P. foetida* var. *intermedia* NIES 512 (**A**), transgenic *S. elongatus* PCC 7942-pYS1C-mtf-mtc (negative control) (**B**) and transgenic *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB (**C**) immunostained with Cnb antibody and gold labeled-secondary antibody. In the magnified views of wild-type *P. foetida* var. *intermedia* NIES 512 and transgenic *S. elongatus* PCC 7942-pYS1C-cnbA-mtf-mtc-cnbB, electron-dense gold particles (indicated by white color arrow heads) shows the presence of Cnb proteins

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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