Genomics and Biochemistry of Metabolic Pathways for the C₁ Compounds Utilization in Colorless Sulfur Bacterium *Beggiatoa leptomitiformis* D-402

Maria V. Orlova¹ · Sergey V. Tarlachkov² · Eugenia I. Kulichenko¹ · Galina A. Dubinina³ · Maria N. Tutukina^{4,5} · Margarita Y. Grabovich¹

Received: 9 March 2018 / Accepted: 24 April 2018 / Published online: 2 May 2018
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Abstract The metabolic pathways of one-carbon compounds utilized by colorless sulfur bacterium *Beggiatoa leptomitiformis* D-402 were revealed based on comprehensive analysis of its genomic organization, together with physiological, biochemical and molecular biological approaches. Strain D-402 was capable of aerobic methylotrophic growth with methanol as a sole source of carbon and energy and was not capable of methanotrophic growth because of the absence of genes of methane monooxygenases. It was established that methanol can be oxidized to CO₂ in three consecutive stages. On the first stage methanol was oxidized to formaldehyde by the two PQQ (pyrroloquinolinequinone)-dependent methanol dehydrogenases (MDH): XoxF and Mdh2. Formaldehyde was

further oxidized to formate via the tetrahydromethanopterin (H₄MPT) pathway. And on the third stage formate was converted to CO₂ by NAD⁺-dependent formate dehydrogenase Fdh2. Finally, it was established that endogenous CO₂, formed as a result of methanol oxidation, was subsequently assimilated for anabolism through the Calvin–Benson–Bassham cycle. The similar way of one-carbon compounds utilization also exists in representatives of another freshwater *Beggiatoa* species—*B. alba*.

Keywords *Beggiatoa leptomitiformis* D-402 · C₁ compounds utilization · Colorless sulfur bacteria · Methylotrophic growth

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12088-018-0737-x>) contains supplementary material, which is available to authorized users.

✉ Margarita Y. Grabovich
grabovichm@gmail.com

¹ Department of Biochemistry and Cell Physiology, Voronezh State University, Universitetskaya pl., 1, Voronezh, Russia 394018

² All-Russian Collection of Microorganisms (VKM), G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region, Russia 142290

³ Federal State Institution “Federal Research Centre “Fundamentals of Biotechnology” of the Russian Academy of Sciences”, Moscow, Russia 117312

⁴ Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region, Russia 142290

⁵ Center for Data-Intensive Biomedicine and Biotechnology, Skolkovo Institute of Science and Technology, Moscow, Russia 143028

Introduction

Strain D-402 belongs to the species *Beggiatoa leptomitiformis* from the family *Beggiatoaceae*, phylum Gammaproteobacteria [1]. In addition to *Beggiatoa*, this family includes two other genera (*Thiomargarita* and *Thioploca*) and 12 candidate lineages [2]. However, to date, only two species from the whole family—freshwater representatives of the genus *Beggiatoa*, *B. alba* [3] and *B. leptomitiformis* [1]—are deposited to the international collections of microorganisms. Genomes of these two strains were sequenced (NCBI reference number NZ_AHMA00000000.1 for *B. alba* B18LD and NZ_CP012373.1 for *B. leptomitiformis* D-402).

Phenotypically, *B. leptomitiformis* D-402 is highly similar to *B. alba* B18LD [1], and both strains have a wide variety of metabolic possibilities: they can use reduced sulfur compounds (thiosulfate, sulfide) [4], molecular hydrogen [1, 5] and a wide range of organic compounds in

energy metabolism [1, 6, 7] and are also able to fix molecular nitrogen [7, 8]. Lithoautotrophic growth with oxidation of reduced sulfur compounds (such as thiosulfate and sulfide) was shown for only one representative of the genus *Beggiatoa*—*B. leptomitiformis* D-402 [4] and was not shown for *B. alba* B18LD [5].

During autotrophic growth, the D-402 strain fixes CO₂ in the Calvin–Benson–Bassham cycle that was shown by measuring activity of the key enzymes, Rubisco (ribulosebiphosphate carboxylase) and phosphoribulokinase, as well as accessory enzyme carboanhydrase [4]. On the basis of amino acid sequence analysis, it was shown that Rubisco from D-402 belonged to the red type form IC [1, 9].

In this work, we present the genome analysis of *B. leptomitiformis* D-402 complemented with physiological, biochemical and molecular biological methods. In particular, we focus on the ability of *B. leptomitiformis* D-402 to convert C₁ compounds: the genomic positioning of genes encoded enzymes for methanol oxidation to CO₂ was revealed, and it was established that endogenous CO₂ from methanol is thereafter assimilated through the Calvin–Benson–Bassham cycle.

Methods

Cultivation

B. leptomitiformis D-402 was grown in the mineral medium of the following composition: 7.3 mM NaNO₃; 1 mM NaH₂PO₄; 0.2 mM CaCl₂·2H₂O; 3.5 mM Na₂SO₄; 1.7 mM KCl; 0.25 mM MgCl₂·6H₂O; pH 7.2. Prior to inoculation, 1 ml of the trace elements and vitamins [10, 11] was added as sterile solutions to 1 litre of the medium in every growth condition and different components were added as sterile solutions to 1 litre of the medium in different growth condition (Table S1).

During microaerobic cultivation, bacteria were grown in 50 ml of liquid medium dispensed into 500 ml vials with butyl rubber stoppers and metal screw caps. Microaerobic conditions were established by filling the vials with freshly boiled sterile medium to capacity, displacing a known amount of liquid with argon, and adding air to 2.5% O₂ concentration in the gas phase. The gas phase was sterilized by filtering through 0.2-μm membranes (Millipore). After 24 h the O₂ content was monitored in the gas phase, using an LHM-80 gas chromatograph, and in the liquid medium by a modified Winkler micromethod [12]. The vials were inoculated with late exponential culture (1 ml). All experiments were performed on the cultures after three to four passages in a relevant medium. In order to avoid a significant decrease in O₂ concentration during growth, the ratio of liquid and gas phases was 1:9.

Genome Analysis

Whole-genome sequence (accession no. NZ_CP012373.1) was taken from our previous work [13]. Additional gene prediction and annotation analysis was performed using RAST [14] and KAAS [15] with default parameters. Functions of the key genes were checked manually with BLAST [16] using the non-redundant protein database. Pairwise sequence alignments were done using the Needleman–Wunsch alignment algorithm (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). These data sources were combined together to identify the metabolic pathways and to reveal gene functions. Circular map of the chromosome was made with DNAPlotter [17].

Gene Expression Analysis

RNA was isolated with ExtractRNA reagent (Evrogen, Russia) according to the manufacturer's protocol. RNA quality was assessed by electrophoresis in 2% agarose gel supplemented with 2.2 M formaldehyde. RNA concentration was measured with the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, USA) on a Qubit 2.0 fluorimeter (Thermo Fisher Scientific, USA). 1000 ng of RNA was then reverse transcribed using M-MuLV (SybEnzyme, Russia) according to the manufacturer's protocol with random hexamer primers. Quantitative RT-PCR was performed on the CFX96 Real-Time System (Bio-Rad, USA) using SYBR Green I master mix (Synthol, Russia), 5 pmol of forward and reverse primer, and 10 ng of cDNA. To find the temperature optimal for amplification, a temperature gradient was first applied. The resulting program included initial denaturation at 95 °C for 3 min; followed by 39 cycles of 20 s denaturation at 95 °C, 20 s primer annealing, and 30 s of elongation at 72 °C. Primers, designed with PrimerBLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), and their annealing temperature are shown in the Table S2.

Cell Lysate Preparation

To prepare cell lysates, the culture was centrifuged for 30 min at 9000 g at 4 °C to collect cells. Pellets were then washed with 0.1 M Tris–HCl buffer (pH 7.5) and centrifuged for 20 min. Cell lysates were obtained by ultrasonic treatment for 2 min on an ice bath using a UZDN-2T sonicator (Akademprigor, Ukraine) at 500 W and 22 kHz. The cytoplasmic fraction was obtained in a supernatant after 30 min centrifugation at 9000g, 4 °C.

Protein concentration was determined by the Lowry method [18]. Prior to analysis, cells were hydrolysed with 1 M NaOH at 90 °C for 10 min.

Enzymatic Activity Assays

Phosphoribulokinase activity was measured using the rate of phosphorus accumulation after alkaline hydrolysis of ribulose-1,5-bisphosphate [19].

Methanol dehydrogenase activity was measured using the modified method of Anthony and Zatman [20, 21].

Formate dehydrogenase activity was assayed by measuring the increase in absorbance at 340 nm due to the production of NADH in the enzymatic reaction [22].

Statistical Analysis

Statistical analysis was performed using statistical variance methods (Microsoft Excel software package). The results are presented as mean standard error of the mean (SEM). The significance of differences between groups was estimated with the Student's *t* test; only statistically significant differences ($p < 0.05$) are discussed in this paper.

Results and Discussion

Genome Analysis

Here, we performed analysis of recently obtained whole genome sequence of *B. leptomitiformis* D-402 [13] to reveal the basis of the C_1 compounds metabolism in the studied strain. This genome is represented by a single circular chromosome, with a length of 4.27 Mbp, and an average G + C content of 40.5% (Fig. 1). A total of 3453 protein-coding genes, of which 1538 CDS are hypothetical proteins, were predicted, as well as 47 tRNAs, 8 ncRNAs, and 6 rRNAs. An average gene length is approximately 1025 bp, and a gene density is 0.86 genes per kb. Genes involved in metabolism of the C_1 compounds on the chromosome are distributed across the genome, without forming a single cluster (track 5 in Fig. 1).

Oxidation of the C_1 Compounds to CO_2

No sign of methanotrophy for *B. leptomitiformis* D-402 was shown: genes of methane monooxygenases were not found in the genome and the growth in the presence of methane was not registered.

Methanol Oxidation to Formaldehyde

In the genome of *B. leptomitiformis* D-402, genes coding for two PQQ-dependent dehydrogenases of methanol/ethanol family, WP_062154153.1 and WP_062149546.1, were found (Table S3).

Fig. 1 Circular map of the *B. leptomitiformis* D-402 chromosome. Two outer tracks represent predicted CDS on forward (purple) and reverse (blue) strands. Third and fourth tracks represent CDS associated with RAST subsystem categories on the forward and reverse strands, respectively. Track five shows genes coding for enzymes of respiratory electron transfer chain and metabolism of one-carbon compounds. The two inner tracks display the GC-plot and the GC-skew (color figure online)

WP_062154153.1 has a high level of identity to XoxF-type methanol dehydrogenase proteins, and WP_062149546.1 has a high level of identity to Mdh2-type methanol dehydrogenase proteins (Table S4).

On the basis of these data, we concluded that the genome of *B. leptomitiformis* D-402 contains the *mdh2* and *xoxF* genes, responsible for synthesis of the PQQ-dependent methanol dehydrogenases. Genes of the *pqqABCDE* operon encoding proteins for biosynthesis of the PQQ coenzyme, which is needed for MDH functioning, were also found (Table S3, Fig. 2a). The amino acid sequences of these proteins were highly homologous to the same proteins from a typical methylotroph *Methylobacterium extorquens* AM1, and the type strain of the genus *Beggiatoa*—*B. alba* B18LD (30–71% and 84–100%, respectively) (Table S5). At the same time, the gene of classical methanol dehydrogenase MxaF was absent in the D-402

Fig. 2 Organization of the genes coding for **a** methanol dehydrogenases and proteins for biosynthesis of coenzyme PQQ: *xoxF*—PQQ-dependent methanol dehydrogenase; *mdh2*—PQQ-dependent methanol dehydrogenase; *pqqA*—pyrroloquinoline quinone precursor peptide PqqA; *pqqB*—pyrroloquinolinequinone biosynthesis protein PqqB; *pqqC*—pyrroloquinolinequinone biosynthesis protein PqqC; *pqqD*—pyrroloquinolinequinone biosynthesis protein PqqD; *pqqE*—pyrroloquinolinequinone biosynthesis protein PqqE **b** enzymes of the H₄MPT-dependent formaldehyde oxidation: *mch*—N(5),N(10)-methenyltetrahydromethanopterin cyclohydrolase (EC 3.5.4.27); *mtdB*—methylene tetrahydromethanopterin dehydrogenase (EC

1.5.99.9); *mptG*—betaribofuranosylaminobenzene 5-phosphate synthase; *fhcB*—formylmethanofuran dehydrogenase subunit B (EC 1.2.99.5); *fhcA*—formylmethanofuran dehydrogenase subunit A (EC 1.2.99.5); *fhcD*—formylmethanofuran-tetrahydromethanopterin N-formyltransferase (EC 2.3.1.101) *fhcC*—formylmethanofuran dehydrogenase subunit C (EC 1.2.99.5); *fae*—formaldehyde activating enzyme **c** formate dehydrogenase: *fdh2A*—formate dehydrogenase subunit alpha (EC 1.2.1.2); *fdh2B*—NAD-dependent formate dehydrogenase beta subunit; *fdh2C*—NAD-dependent formate dehydrogenase gamma subunit; *fdh2D*—NAD-dependent formate dehydrogenase delta subunit

genome as well as in *B. alba* B18LD genome. In the previous paper devoted to the description of methylotrophic growth of *B. alba* [23], only XoxF-type methanol dehydrogenases were detected. However, our genome analysis revealed the gene for Mdh2 methanol dehydrogenase in *B. alba* (WP_002685746.1). This is a direct evidence that two species of freshwater *Beggiatoa* have the same type of methanol dehydrogenases.

Formaldehyde Oxidation to Formate and CO₂

The genome of D-402 contained genes, encoding enzymes of the H₄MPT pathway of formaldehyde oxidation to formate, which did not form a single cluster but were located at a large distance from each other (Fig. 2b). In contrary, genes of the NAD⁺-dependent formate dehydrogenase responsible for further formate oxidation to CO₂, *fdh2ABCD*, were located in a single cluster (Table S3, Fig. 2c).

The amino acid sequences of enzymes responsible for formaldehyde oxidation to CO₂ were 30–70% identical to the same enzymes from a typical methylotroph *M. extorquens* AM1, and 66–93% identical to that from the type strain of the genus *Beggiatoa*—*B. alba* B18LD (Table S6).

Growth of *B. leptomitiformis* D-402 in the Presence of Methanol

B. leptomitiformis D-402 demonstrated stable growth during several passages aerobically with methanol as a sole source of carbon and energy under rubber stoppers. Protein increment was 12 mg l⁻¹.

It is known that highly active XoxF-type methanol dehydrogenases require for their activity the presence of rare-earth metals—lanthanoids [24–26]. Addition of lanthanoids into the nutrient medium thus may stimulate the growth of methylotrophs. In our case, addition of lanthanum chloride (III) really led to the 2.75 fold biomass increment, with the protein increment of 33 mg l⁻¹.

As shown in our earlier study, during lithoautotrophic growth thiosulfate is oxidized to S⁰, intracellularly stored in the periplasm, and SO₄²⁻ [1] (Fig. 3a). After 2–3 passages of bacteria in the medium with methanol but without thiosulfate, sulfur globules disappeared (Fig. 3b), bacteria passed from lithotrophic growth to methylotrophic growth.

Thus, the protein increment for methylotrophically growing *B. leptomitiformis* D-402 is comparable to the protein increment during its lithoheterotrophic growth [27] when abundant biomass is formed.

Fig. 3 Morphology of *B. leptomitiformis* D-402 under different cultivation conditions: **a** chemolithoautotrophic growth with thiosulfate, **b** methylotrophic growth. 10 μ m scale. S^0 —elemental sulfur

Methanol Dehydrogenase and Formate Dehydrogenase Activity

Activity of methanol dehydrogenase (total activity of XoxF and Mdh2) and formate dehydrogenase was 35 ± 1.75 and $0.07 \pm 0.003 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$, respectively. In the presence of La (III), the total activity of methanol dehydrogenase and formate dehydrogenase increased 2.6 and 3.5 fold, respectively.

We suspect that the increase of the XoxF activity in the presence of La (III) led to the increased concentration of the final reaction product, formaldehyde, leading consequently to the increase in the amount of intermediate H_4 MPT pathway products. This, in turn, led to the increased formate concentration. So, addition of La (III) run the mechanism of the cascade increase of final reaction products and, consequently, the increase in efficiency of methanol assimilation.

Expression Level of the *mdh2* and *xoxF* Genes

qRT-PCR (Fig. 4a) demonstrated 1.6 and 2 fold increase in the *mdh2* and *xoxF* expression during methylotrophic growth as compared to lithoheterotrophic growth. The expression level of the *xoxF* gene during methylotrophic growth with La (III) was 2.2 fold higher as compared to methylotrophic growth without La (III) (Fig. 4a). Homogeneity of the PCR products was confirmed by electrophoresis in 2% agarose gel (Fig. 4b). These results are in line with the earlier described influence of rare-earth metals on activity of the XoxF-type MDH [24–26].

Carbon Assimilation from the C_1 Compounds for Anabolism

Three main pathways of carbon assimilation for anabolism during methylotrophic growth are known for bacteria: the RuMP (ribulose monophosphate) cycle, the serine cycle, and the Calvin–Benson–Bassham cycle [28].

Genes, encoded the key enzymes of the RuMP cycle, hexulose-6-phosphate synthase (*hxlA*) and phosphohexulose isomerase (*hxlB*), and several enzymes of the serine cycle, serine-glyoxylate aminotransferase (*sga*), glycerate kinase (*gck*) and malyl-CoA lyase (*mcl*) were not found in the genome of *B. leptomitiformis*. Thus, these cycles presumably can not function in D-402.

However, the complete set of genes for the Calvin–Benson–Bassham cycle was revealed (Table S3). It should be noted that the gene encoding sedoheptulose-1,7-biphosphatase is absent in the *B. leptomitiformis* genome, but this enzyme can be substituted by fructose-1,6-biphosphatase, taking into account its double specificity to sugars in bacteria [29]. Figure 5 demonstrates that the

Fig. 4 **a** Expression of the *prkB*, *rbcL*, *xoxF* and *mdh2* genes during lithoheterotrophic, methylotrophic aerobic without La (III), and methylotrophic aerobic growth with La (III). Error bars were calculated based on three independent experiments. **b** Agarose gel electrophoresis product quality verification after amplification of the

prkB, *rbcL*, *xoxF* and *mdh2* gene fragments with the appropriate primer pairs (Table S2). M—DNA Molecular weight ladder; 1—*prkB*, 2—*rbcL*, 3—*xoxF*, 4—*mdh2* (expected product lengths are 208, 258, 339 and 334 bp, respectively). 16S rRNA and *gyrB* genes were used as reference

Fig. 5 Organization of the genes coding for the enzymes of the Calvin–Benson–Bassham cycle: *pgk*—Phosphoglycerate kinase (EC 2.7.2.3); *fbaB*—Fructosebisphosphate aldolase class I (EC:4.1.2.13); *gapA*—NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13)/NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); *tktA*—Transketolase (EC 2.2.1.1); *rpiA*—Ribose 5-phosphate isomerase B (EC 5.3.1.6); *rpe*—Ribulose-phosphate 3-epimerase (EC:5.1.3.1); *tpiA*—Triosephosphate

isomerase (EC 5.3.1.1); *cbbR*—RuBisCO operon transcriptional regulator CbbR; *rbcL*—Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39); *rbcS*—Ribulose bisphosphate carboxylase small chain (EC 4.1.1.39); *cbbX*—Probable RuBisCo-expression protein CbbX; *fbp*—Fructose-1,6-bisphosphatase, type I (EC 3.1.3.11); *prkB*—Phosphoribulokinase (EC 2.7.1.19) and *fbaA*—Fructose-bisphosphate aldolase class II (EC 4.1.2.13)

genes of the Calvin–Benson–Bassham cycle do not form a single cluster.

Using cultural and biochemical approaches, it was previously shown that *B. leptomitiformis* D-402 can grow autotrophically only in microaerobic conditions [4]. As such, to confirm functioning of the Calvin–Benson–Bassham cycle, we measured expression level of genes coding for its key enzymes, *prkB* and *rbcL*, under microaerobic growth. qRT-PCR demonstrated that under microaerobic lithoautotrophic conditions the *prkB*- and *rbcL*-mRNA levels was 3 and 9.5 fold, and 4 and 13 fold higher than that in lithohetero- and organoheterotrophic conditions, respectively (Fig. 6).

Methylotrophic aerobic growth was discovered, so it was interesting to understand the mechanism of CO₂ assimilation during aerobic growth. For this purpose, we assessed the activity of one of the key enzymes for the CO₂ assimilation by the Calvin–Benson–Bassham cycle, phosphoribulokinase, during methylotrophic growth. To confirm the above assumption about the influence of La (III) on the whole pathway of C₁ compounds conversion we measured the activity of phosphoribulokinase with La (III) and without it. During methylotrophic growth in aerobic

conditions with La (III), phosphoribulokinase activity was 1.6 fold higher than that without La (III), and had a value of $2.1 \pm 0.1 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$. The activity of phosphoribulokinase during aerobic methylotrophic growth is comparable to the activity of this enzyme during lithoautotrophic microaerobic growth with thiosulfate [4], which indirectly supports the functioning of Calvin–Benson–Bassham cycle during methylotrophic growth.

To further confirm CO₂ assimilation by the Calvin–Benson–Bassham cycle, we used qRT-PCR analysis, which demonstrated that the *prkB*- and *rbcL*-mRNA levels under methylotrophic aerobic conditions were approximately 10 fold higher than that in lithoheterotrophic conditions (Fig. 4).

So we showed that this strain is capable of methylotrophy in aerobic conditions, and can oxidize methanol to CO₂ in three stages: methanol is oxidized to formaldehyde by the two PQQ-dependent methanol dehydrogenases, XoxF and Mdh2; formaldehyde is further oxidized to formate via the H₄MPT pathway, and formate is converted to CO₂ by NAD⁺-dependent formate dehydrogenase. It was shown that the C₁ compounds for anabolic processes are obtained from CO₂ assimilated by the Calvin–Benson–Bassham cycle.

The Calvin–Benson–Bassham cycle in *B. leptomitiformis* D-402 provides conversion of CO₂ during methylotrophic and lithoautotrophic growth. In the latter case, this cycle functions only in microaerobic conditions allowing assimilation of exogenous CO₂ [4]. In contrast, during methylotrophic growth, it functions in aerobic conditions using exogenous and endogenous CO₂, which is produced because of methanol oxidation (Fig. 7).

The comparison of metabolic pathways functioning during methylotrophic growth of *B. leptomitiformis* D-402 and other representatives of the freshwater *Beggiatoa*, revealed their similarity with the C₁ compounds assimilation for anabolism in *B. alba*. It should be noted that *B. alba* does not have any of the key enzymes of the RuMP cycle, and also lack several enzymes of the serine cycle: seryne-glyoxylate aminotransferase (Sga), glycerate kinase (Gck) and malyl-CoA lyase (Mcl). The same is also true for

Fig. 6 Expression of the *prkB* and *rbcL* genes during organoheterotrophic, lithoheterotrophic, and lithoautotrophic growth in microaerobic conditions. Error bars were calculated based on three independent experiments. 16S rRNA and *gyrB* genes were used as reference

Fig. 7 The ways of catabolism and anabolism in *B. leptomitiformis* D-402^T under chemolithoautotrophic microaerobic and methylotrophic aerobic cultivation

B. leptomitiformis. This may indicate that the CO₂ assimilation in *B. alba* during methylotrophic growth may also be implemented through the Calvin–Benson–Bassham cycle.

Electron Transport Chain

The genome of *B. leptomitiformis* D-402 encodes a full set of genes for the components of electron transport chain (Table S3, cyan bars on the track five in Fig. 1), which makes possible respiration of this strain on different energy substrates including methanol and other C₁ compounds.

Taking together, the ability of *B. leptomitiformis* D-402 for growth with methanol as a sole source of carbon and energy was shown the first time. Methanol is oxidized to CO₂ in three stages. Methylotrophy has been shown for only one representative of the genus *Beggiatoa*—*B. alba* [23]. However, the way of further assimilation of the C₁ compounds for *B. alba* anabolism has never been revealed before. Sequencing of genomes for the representatives of *Beggiatoa* genus facilitated greatly the task of metabolic pathways identifying. Here, we show that during methylotrophic growth, representatives of the genus *Beggiatoa* can assimilate the CO₂ by the Calvin–Benson–Bassham cycle. A comprehensive study of methylotrophy in lithotrophic colorless sulfur bacteria *B. leptomitiformis* D-402 expands our knowledge of the taxonomic composition of methylotrophs and suggests that lithotrophic sulfur

bacteria, forming abundant mats in nature, can play an important role in the control of the content of toxic compounds, such as methanol and hydrogen sulfide, in the biosphere.

Acknowledgements The authors would like to express sincere gratitude to Alexey Fomenkov, Tamas Vincze, Brian P. Anton and Richard J. Roberts for their help with genome sequencing.

Funding This work was supported by Russian Foundation for Basic Research (Grant 18-04-00556).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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