
EXPERIMENTAL ARTICLES

Nitrogen Metabolism of an Anoxygenic Filamentous Phototrophic Bacterium *Oscillochloris trichoides* Strain DG-6

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Received February 21, 2021; revised March 22, 2021; accepted March 24, 2021

Abstract—The possible nitrogen sources for *Osc. trichoides* DG6, a typical strain of the *Oscillochloridaceae* family, are ammonium, N₂, glutamate, asparagine, glycine, and glutamine. The assimilation of molecular nitrogen occurs with the participation of nitrogenase, the structural gene of which, *nifH*, is located in the gene cluster which also includes the genes of the *nifD* and *nifK* nitrogenase subunits and the auxiliary *nifB* gene. Considering that *nifH*BDK clusters have been also annotated in the genomes of other members of the *Oscillochloridaceae* family, including uncultured and candidate taxa, it can be assumed that the ability to fix nitrogen is a property immanent for this entire family. The pathways for assimilating ammonium in the cells grown using different nitrogen sources may differ. *Osc. trichoides* DG6 growing in a medium containing ammonium assimilated it with the participation of glutamate dehydrogenase, which is determined by a single gene. The expression product of this gene has dual functionality and can be used to implement the reaction with both NAD and NADP. With the growth of *Osc. trichoides* DG6 on a medium with glutamate as the only nitrogen source all the enzymes necessary for the implementation of the GS-GOGAT pathway were found in the cells. However, for the glutamine synthetase reaction, ammonium, which was absent in the growth medium, was required. The source of ammonium may be glutamate metabolized through glutamate dehydrogenase.

Keywords: anoxygenic filamentous phototrophic bacteria, *Oscillochloris*, nitrogen metabolism, assimilation of N₂ and ammonium

DOI: 10.1134/S0026261721040068

All currently known anoxygenic filamentous phototrophic bacteria (AFPBs) belong to the phylum *Chloroflexi*. Members of the order *Chloroflexales* form a monophyletic lineage that includes the families *Chloroflexaceae*, *Roseiflexaceae*, and *Oscillochloridaceae*. The AFPBs classified into these families differ in terms of photosynthetic machinery composition, capacity for autotrophy, and growth temperature. Members of these families also differ in their nitrogen metabolism (Hanada, 2014).

There is no evidence that any representatives of the phototrophic thermophilic family *Roseiflexaceae* are capable of utilizing molecular nitrogen as a nitrogen source (Thiel et al., 2017). However, the genomes of *Roseiflexus* sp. RS1 and *Roseiflexus castenholzii* contain a cluster of four co-located *nifH*BDK genes that presumably code for the structural genes of the Mo nitrogenase. It was suggested that the incapacity of these bacteria to grow under diazotrophic conditions results from a lack of supplementary genes that are normally implicated in the biosynthesis and maturation of the functional apoprotein of the nitrogenase

(van der Meer et al., 2010; Klatt et al., 2013; Thiel et al., 2017; Nishihara et al., 2018).

Representatives of the family *Chloroflexaceae* can utilize ammonium and amino acids, such as alanine, glutamate, glutamine, aspartate, glycine, and serine, but not nitrates as the sole nitrogen source (Heda and Madigan, 1986; Hanada, 2014). Nitrogen-fixing capacity and nitrogenase activity were not detected in four *Chloroflexus aurantiacus* strains which was correlated with their thermophily (Heda and Madigan, 1986; Hanada, 2014). Subsequently, these data were confirmed by analyzing the *Cfl. aurantiacus* genome that lacked the structural genes of nitrogenase and nitrate reductase (Tang et al., 2011).

The photoautotrophic bacteria of the family *Oscillochloridaceae*, which are the main subject of the present work, are mesotrophic microorganisms with a growth optimum of 28–30°C (Keppen et al., 1993). The main nitrogen sources for the growth of *Oscillochloris trichoides* DG-6, the type strain of the family *Oscillochloridaceae*, are ammonium and the amino acids asparagine, glycine, glutamate, and gluta-

Table 1. Reactions and the N metabolism pathway enzymes of *Osc. trichoides* DG-6 that catalyze them; their activities were measured in this work

Enzyme	Gene locus tag in the genome (locus_tag)	Reaction
Nitrogenase EC 1.18.6.1	OSCT_0062 OSCT_0063 OSCT_0064 OSCT_0065	$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$
Glutamate dehydrogenase (GDH) EC 1.4.1.2; EC 1.4.1.4	OSCT_0145	$Glutamate + NAD(P)^+ + H_2O \rightarrow \alpha\text{-Ketoglutarate} + NAD(P)H + NH_3$
Glutamine synthetase (GS) EC 6.3.1.2	OSCT_1286	$Glutamate + NH_4 + ATP \rightarrow Glutamine + ADP + P_i$
Glutamate synthase (GOGAT) EC 1.4.1.13	OSCT_0551	$2Glutamate + NADP^+ \rightarrow Glutamine + \alpha\text{-Ketoglutarate} + NADPH$
Glutamate–oxaloacetate transaminase (GOT) EC 2.6.1.1	OSCT_0733 OSCT_1058 OSCT_1297 OSCT_3177	$Aspartate + \alpha\text{-Ketoglutarate} \rightarrow Oxaloacetate + Glutamate$
Glutamate–pyruvate transaminase (GPT) EC 2.6.1.2	OSCT_0566	$\alpha\text{-Ketoglutarate} + Alanine \rightarrow Glutamate + Pyruvate$

mine (Keppen et al., 1989). A distinctive feature of *Osc. trichoides* that sets it apart from bacteria belonging to the genera *Roseiflexus* and *Chloroflexus* is its ability to utilize N_2 as the sole nitrogen source for its growth (Keppen et al., 1989). Subsequently, amplification and sequencing of the *nifH* gene encoding the nitrogenase, the key enzyme of the nitrogenase complex, was carried out; the gene is located on the DNA matrix of three *Oscillochloris* strains, including *Osc. trichoides* DG-6 (Tourova et al., 2006).

The goal of the present work was comparative analysis of the genome data that are concerned with the putative nitrogen metabolism processes in cultivable AFPBs of the order *Chloroflexales* and experimental confirmation of the implementation of these processes by the bacteria of the strain *Osc. trichoides* DG-6.

MATERIALS AND METHODS

Bacteria and cultivation conditions. The research subject was the anoxygenic filamentous phototrophic bacterium (AFPB) *Osc. trichoides* DG-6. Its cultures were grown in a modified DGN medium with a vitamin complex, 0.1% acetate, 0.1% bicarbonate, and 0.1% sulfide (Keppen et al., 1994). The bacterium was cultivated under anaerobic conditions in the light with a magnetic stirrer (300 rpm) at a temperature of 28–30°C and a light intensity of 2000 lx in 500-mL screw-top vials. If N_2 was used as a nitrogen source, the flasks

contained 300 mL of the medium and molecular nitrogen as the gas phase. The flasks were completely filled with the medium when ammonium or glutamate were used as nitrogen sources.

Obtaining cell extracts. To obtain cell-free extracts of a culture at the end of the exponential growth phase, the culture was centrifuged (9000 rpm at 6–8°C) and washed with 0.05 M Tris-HCl buffer (pH 7.5); the cells were disintegrated with an X press (LKB, Sweden) at an excess pressure of 10 t/cm². Intact cells and large fragments were separated by centrifugation at 35000 g for 1 h. The resulting supernatant was used to determine enzymatic activity.

Determining the enzymatic activity. The reactions of the N metabolism pathways in *Osc. trichoides* DG-6 and the catalyzing enzymes whose activities were measured in this work are listed in Table 1. The enzymatic activities were determined with a Hitachi 200 spectrophotometer (Hitachi, Japan) from NAD(P)H oxidation and NAD(P) reduction at 340 nm.

The reaction mixtures for determining enzyme activities contained the following:

(1) for glutamate dehydrogenase (GDH): ketoglutarate, 5×10^{-3} M; NH_4Cl , 4×10^{-2} M; NAD(P)H, 2.5×10^{-4} M; Tris-HCl (pH 7.5), 5×10^{-2} M;

(2) for glutamate synthase (GOGAT): ketoglutarate, 5×10^{-3} M; glutamine, 5×10^{-3} M; NAD(P)H; 2.5×10^{-4} M; Tris-HCl (pH 7.5); 5×10^{-2} M;

(3) for glutamate–oxaloacetate transaminase (GOT): NAD, 2.5×10^{-4} M; ketoglutarate, 4×10^{-3} M; aspartate, 4×10^{-3} M; phosphate buffer (pH 7.4), 0.1 M; malate dehydrogenase, 1.25 mg/mL;

(4) for glutamate–pyruvate transaminase (GPT): NAD, 2×10^{-4} M; ketoglutarate, 4×10^{-3} M; phosphate buffer (pH 7.5), 0.1 M; alanine, 8×10^{-3} M; lactate dehydrogenase, 1.25×10^{-3} mg/mL;

(5) glutaminase (GA): glutamine, 4×10^{-3} M; Tris-HCl (pH 7.5), 5×10^{-2} M; glutamate dehydrogenase, 0.15 U/mL.

Glutamine synthetase (GS) activity was determined as was described earlier (Ivanovsky and Khatipov, 1994).

All reaction mixtures were supplemented with cell extract containing 1.0–2.0 mg/mg protein. Proteins were determined by the Lowry method.

Bioinformatic analysis. Reconstruction of the possible pathways of nitrogen metabolism was carried out by comparing the genomes of cultured AFPB strains of the order *Chloroflexales*, using NCBI (<https://www.ncbi.nlm.nih.gov/genome/>), PATRIC (<https://www.patricbrc.org>), and KEGG (<https://www.genome.jp/kegg/pathway.html>) online services. Comparison of the *nif* gene clusters was performed using the Gene Graphics (<https://katlabs.cc/genegraphics/app>) online service.

RESULTS AND DISCUSSION

Potential functional characteristics of nitrogen metabolism of the cultivable AFPB strains of the order *Chloroflexales* based on comparison of their genomes. Gene set enrichment analysis for nitrogen metabolism and for alanine, aspartate and glutamate metabolism was performed using the KEGG database for the genomes of cultivable strains of the genera *Oscillochloris*, *Chloroflexus*, and *Roseiflexus* (Fig. S1a, S1b).

According to the genome data for all strains to be compared, the heat maps of the putative nitrogen metabolism genes contained glutamate conversion enzymes, such as glutamate synthase (GOGAT) (NAD(P)H) (EC 1.4.1.13); glutamine synthetase (GS) (EC 6.3.1.2), and glutamate dehydrogenase (GDH) in two functional variants that interact with NADH (EC 1.4.1.2) and NAD(P)H (EC 1.4.1.4), respectively. The same enzymes were present in all tested strains on the heat map of the enzymes of alanine, aspartate, and glutamate metabolism.

The presence of other putative nitrogen metabolism enzymes markedly varied, depending on the taxonomic affiliation of the tested strains. Nitrogenase (EC 1.18.6.1) that is responsible for molecular nitrogen fixation has only been annotated in the genomes of

Table 2. Growth of *Osc. trichoides* DG-6 on media with various nitrogen sources (mg of protein per 1 L)*

Nitrogen source	Biomass accumulation
Ammonium	23.3
Glutamate	22.2
Aspartate	26.0
Asparagine	11.2
Urea	23.4
N ₂	1.2

* The cultivation time was 48 h.

Oscillochloris and *Roseiflexus* strains, similar to carbonic anhydrase (EC 4.2.1.1). Hydroxylamine reductase (EC 1.7.99.1) has only been annotated for *Oscillochloris* strains, and nitrate reductase (EC 1.7.99.4), for *Roseiflexus* strains. Most enzymes of alanine, aspartate, and gluconate metabolism have been annotated in the genomes of all tested strains; however, alanine dehydrogenase (EC 1.4.1.1) has only been annotated in the strains of *Roseiflexus* and alanine transaminase (EC 2.6.1.2) in the strains of *Oscillochloris*.

The absence of the genes that determine the enzymes of assimilative and dissimilative nitrate reduction in the genomes of cultivable *Chloroflexales* and *Oscillochloris* strains accounts for the earlier experimental data on the incapacity of the AFPBs of these genera to grow on media with oxidized nitrogen compounds. Nevertheless, this confirms the data on utilization of reduced nitrogen compounds (ammonium, urea, glutamate, and a number of amino acids) for the growth of strains belonging to the genera *Chloroflexus* and *Oscillochloris* (Heda and Madigan, 1986; Keppen et al., 1993, 2000). The results of comparative genome analysis were consistent with the earlier data on the incapacity of the AFPBs of the genus *Chloroflexus* to fix molecular nitrogen (Heda and Madigan, 1986) and, conversely, the capacity for nitrogen fixation in the AFPBs of the genus *Oscillochloris* (Ivanovsky and Khatipov, 1994; Keppen et al., 1989, 2000).

Nitrogen sources utilized by *Osc. trichoides* DG-6. Nitrogen sources for *Osc. trichoides* DG-6 included ammonium, N₂, and the amino acids asparagine, glycine, glutamate, and glutamine (Table 2).

Assimilation of molecular nitrogen by *Osc. trichoides* DG-6. The presence of the *nifH* gene that encodes the key enzyme of molecular nitrogen fixation was earlier revealed in experiments on amplifying the gene not only in the type strain DG-6, but also in several closely related strains also classified into the species *Osc. trichoides* (Tourova et al., 2006). The functional activity of nitrogenase was confirmed by demonstrating the growth of the culture in the presence of N₂ as the sole nitrogen source (Keppen et al., 1989). In the

Table 3. Nitrogenase activity in *Osc. trichoides* DG-6 (nmoles/min/mg of protein)

Enzyme/growth substrate in the medium	Glutamate, 0.1%	NH ₄ ⁺ , 0.1% ⁺	N ₂
Nitrogenase	0.9	0.0	3.2

present work, nitrogenase activity was observed during the growth of strain DG-6 on N₂ by detecting the ability of the cell suspension to reduce acetylene; this activity was repressed by adding reduced nitrogen compounds (Table 3). The nitrogenase enzyme is present on the nitrogen metabolism map in the KEGG database, which is in accordance with the results of analysis of the genome of *Osc. trichoides* DG-6 (Fig. S2a). Based on these data, the previously detected nitrogenase reductase gene (*nifH*) forms a part of the gene cluster (OSCT_0062, OSCT_0063, OSCT_0064, OSCT_0065) that also includes the genes of nitrogenase subunits (*nifD* and *nifK*) and the auxiliary *nifB* gene (Fig. 1). Therefore, *Osc. trichoides* DG-6 is currently the only representative of the order *Chloroflexales* whose capacity for nitrogen fixation has been demonstrated using genome data as well as experimentally. However, the genome data indicate that the recently isolated AFPB species “*Candidatus Oscillochloris kuznetsovii*” and “*Candidatus Oscillochloris fontis*,” “*Candidatus Chloroploca asiatica*” and “*Candidatus Viridilinea mediisalina*” may be capable of diazotrophy, due to the presence of the nitrogenase gene cluster (Grouzdev et al., 2018, 2019; Gaisin et al., 2019a, 2019b, 2020) that is homologous to the *nifHBDK* cluster of the strain *Osc. trichoides* GD-6. Taking into account that the *nifHBDK* clusters were also annotated in the genomes of other represen-

tatives of the family *Oscillochloridaceae*, including uncultured and candidate species, the nitrogen fixation capacity is presumably a feature that is characteristic of the whole family.

Nonetheless, the genomes of cultivable AFPB strains of the genus *Roseiflexus* also contain a nitrogenase gene cluster that is structurally similar to the *nifHBDK* cluster of representatives of the genus *Oscillochloris*, except for the gene overlap detected in them (Fig. 1). No data on the nitrogenase functional activity in cultivable *Roseiflexus* spp. strains are available. The type strain of *Rof. castenholzii* cannot grow on N₂ as the only nitrogen source (Thiel et al., 2017). However, the aforementioned suggestion that this is due to a lack of other genes, *nifE* and *nifN* that determine the minimum set of auxiliary proteins for nitrogenase maturation (Dos Santos et al., 2012) becomes dubious, considering the functional activity of an analogous *nifHBDK* cluster in the strains of *Oscillochloris*. Presumably, the gene overlap in the cluster indicates a mutation process involving a loss of the functional activity of nitrogenase genes in the strains of the genus *Roseiflexus*, due to their adaptation to thermophilic environmental conditions.

The experimental evidence of existence of an extremely simplified but still functional cluster of nitrogenase genes might be of paramount importance

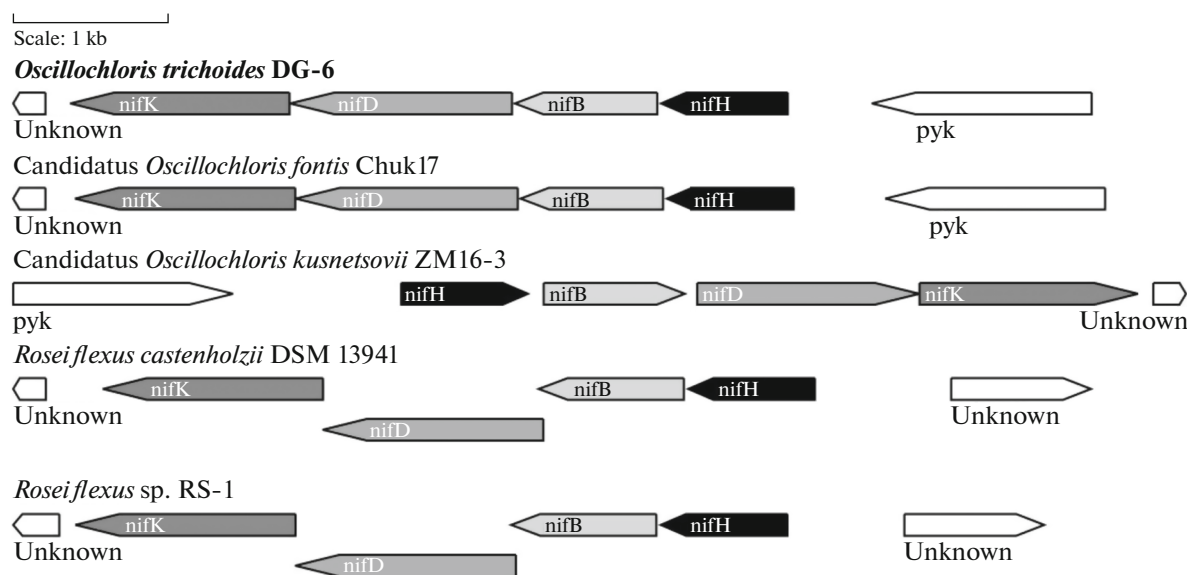


Fig. 1. Structure of the genome clusters containing annotated *nif* genes in members of the families *Oscillochloridaceae* and *Roseiflexaceae*.

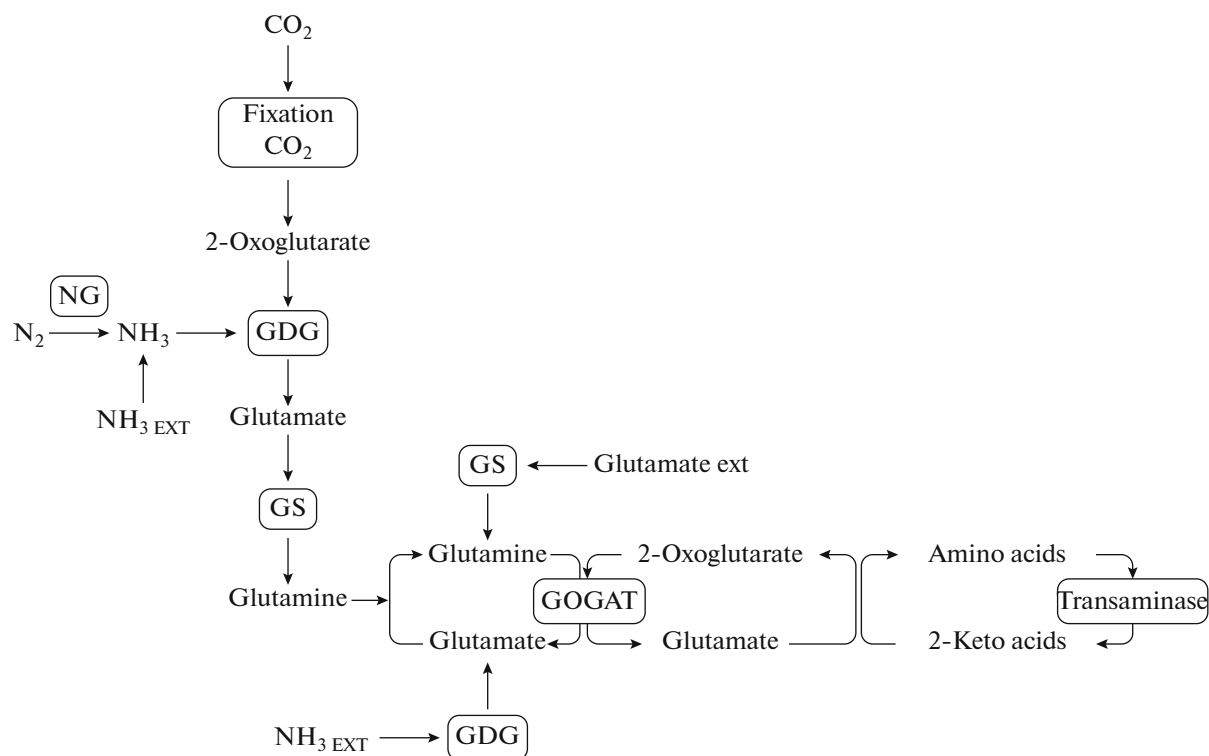


Fig. 2. Nitrogen metabolism in *Osc. trichoides* DG-6 utilizing various nitrogen sources.

for potential biotechnological developments regarding the implantation of these genes into a plant genome.

Assimilation of reduced nitrogen compounds by *Osc. trichoides* DG-6. Similar to most phototrophic bacteria, *Osc. trichoides* DG-6 grows optimally on media with reduced nitrogen compounds (Table 2). The maps of pathways of nitrogen, alanine, aspartate, and glutamate metabolism based on analyzing the *Osc. trichoides* DG-6 genome in the KEGG database are shown in Figs. S2a, S2b. The activities of respective enzymes in *Osc. trichoides* DG-6 grown in media with various nitrogen sources were assessed in the present work (Table 2). The results obtained indicate that, despite the presence of all ammonium assimilation pathways-determining genes in the tested genome, the implementation of these pathways in the cells grown with different nitrogen sources may be different (Fig. 2). Similar to other AFPBs, ammonium can be incorporated in *Osc. trichoides* DG-6 metabolism via the GS–GOGAT pathway. According to genomic data, the enzymes of this pathway, glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.1.13), encoded by one gene each (OSCT_1286 and OSCT_0551, respectively), are present on the metabolism maps in question (Fig. S2a, S2b). However, GS activity was only detectable in the medium with glutamate (Table 4) but not on the media with ammonium, unlike *Cfl. aurantiacus*, for which the measured activity of this enzyme was even

higher in the medium with ammonium than in the medium with glutamate (Kaulen and Klemme, 1983).

According to the data obtained in the present work, *Osc. trichoides* strain DG-6 growing in the medium with ammonium uses the ammonium assimilation pathway involving glutamate dehydrogenase (Fig. 2). Glutamate dehydrogenases (GDHs: EC 1.4.1.2 and EC 1.4.1.4) are encoded by a single gene (OSCT_0145) that has been annotated in the *Osc. trichoides* DG-6 genome (Figs. S2a, S2b). The

Table 4. Enzyme activities involved in ammonium assimilation in *Osc. trichoides* DG-6 (nmol/min mg of protein)

Enzyme/nitrogen source	Glutamate, 0.1%	NH ₄ ⁺ , 0.1% ⁺
GDH NADH	3.2	6.5
GDH NAD(P)H	3.2	2.0
GOGAT NADH	1.9	2.0
GOGAT NAD(P)H	0.9	2.0
GS	20.2	0.0
GA	4.0	4.9
GOT	42.1	70.5
GPT	47.3	7.8

The enzyme activities were not measured in the cells growing in the medium with molecular nitrogen because insufficient biomass was produced under these conditions.

product of the gene is characterized by dual substrate specificity: it can use both NAD and NADP for carrying out the process. A twofold increase in activity of the NAD-dependent enzyme isoform in the extracts of ammonium-grown cells may indicate its preferential involvement in assimilating the substrate (Table 4).

If *Osc. trichoides* DG-6 grows in a medium with glutamate as the sole carbon source, its cells contain all the enzymes that are necessary for implementing the GS–GOGAT pathway (Table 4). However, carrying out the glutamine synthetase reaction requires ammonium that is absent from the medium. The glutamate dehydrogenase reaction can provide the cells with ammonium (Fig. 2), since the activities of both GDH isoenzymes were detected in the cells grown in the medium with glutamate (Table. 4).

Apparently, the GS–GOGAT pathway in *Osc. trichoides* is also implemented during nitrogen fixation. However, additional research is necessary for verifying this statement.

The alanine dehydrogenase gene (ADH, EC 1.4.1.1) in *Osc. trichoides* has not been annotated, which rules out the involvement of this enzyme in assimilating ammonium in this AFPB, similar to *Cfl. aurantiacus*. The gene was not annotated in the *Cfl. aurantiacus* genome and the enzyme's activity was not detected (Kaulen and Klemme, 1983). However, the alanine transaminase gene (GPT, EC 2.6.1.2) was annotated in *Osc. trichoides*, which sets *Osc. trichoides* apart from other cultivated strains of the order *Cloroflexales*. Its activity was higher in the medium with glutamate than with ammonium, similar to another enzyme, aspartate transaminase (GOT, EC 2.6.1.1) (Table 4). The high activities of transaminating enzymes are in conformity with the capacity of *Osc. trichoides* to utilize amino acids as nitrogen sources for its growth.

The data obtained in this work on the patterns of nitrogen metabolism of *Osc. trichoides* DG-6 with various nitrogen sources are summed up in Fig. 2.

The conclusion may be drawn that integrated research on the genetic and enzymatic aspects of organic nitrogen metabolism make it possible to elucidate the ecological and evolutionary features of AFPBs of the family *Oscillochloridaceae* and assessing the applicability of their metabolic potential in biotechnology.

FUNDING

This work was partially supported by the Russian Foundation for Basic Research (grant no. 20-54-12031 NNIO_a).

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving ani-

mals or human participants performed by any of the authors.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at <https://doi.org/10.1134/S0026261721040068>.

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Translated by A. Oleskin