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EXPERIMENTAL ARTICLES

Diversity of Copper Proteins and Copper Homeostasis Systems in *Melioribacter roseus*, a Facultatively Anaerobic Thermophilic Member of the New Phylum *Ignavibacteriae*

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Abstract—The genome of *Melioribacter roseus*, one of the two members of the recently described phylum *Ignavibacteriae,* was screened for genes encoding proteins associated with copper transport or containing copper as a cofactor, and the effect of Cu^{2+} concentration in the medium on *M. roseus* growth was investigated. Genomic analysis revealed a variety of copper-containing oxidoreductases in this facultative anaerobe. Three ATPases responsible for copper transport were identified. One of them (MROS_1511) is probably involved in the assembly of copper-containing cytochrome *c* oxidase, while two others (MROS_0327 and MROS_0791) probably carry out a detoxification function. The presence of several copper-containing oxidoreductases and copper homeostasis systems in *M. roseus* is in agreement with the previously hypothesized origin of the phylum *Ignavibacteriae* from an aerobic ancestor common with *Bacteroidetes* and *Chlorobi*.

Keywords: genomic research, copper proteins, copper homeostasis, copper ATPases, *Melioribacter roseus, Ignavibacteriae*

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Copper, along with iron and zinc, is among the most common metals that occur in metalloproteins of prokaryotic and eukaryotic cells [1]. Copper is contained, as a cofactor, in such prokaryotic proteins as cytochrome oxidases, superoxide dismutases, amine oxidases, methane monooxygenases, and other copper oxidases and laccases. Geochemical calculations show that, under anaerobic conditions, copper is, for the most part, present in the form of sulfides with an extremely low solubility, which are unavailable for living cells [2]. Presumably, the copper utilizable by living cells appeared in significant quantities in the ocean only after its oxygenation, which started between two and three billion years ago. Therefore, copper is envisioned as a "modern" metal [3]. The analysis of the complete genomes of bacteria and archaea demonstrated that most of anaerobes do not use Cu as an enzyme cofactor. In contrast, genes encoding copper proteins are widespread in the genomes of aerobes [1]. Thus, genomic research has confirmed the idea that protein-encoded geochemical environmental signals are conserved on the geological time scale, and the investigation of them can help us retrace the time course of some evolutionary events in living organisms [2].

Although copper occurs in the composition of biological molecules, it is toxic for living cells at high concentrations (>1 mg/L). Presumably, the biochemical mechanisms that protect the cell from copper ions emerged in the evolution much earlier than the first copper proteins [3, 4]. The main mechanism of copper detoxification involves pumping excess copper out of the cytoplasm by copper ATPases of the P type [5]. Along with the ATPases, other enzymes were revealed in proteobacteria. They include RND efflux transporters, which usually transfer copper from the periplasmic space, sequestering copper oxidases, and polyphosphate-forming polyphosphate kinases. Unlike copper ATPases, RND transporters extrude the ions of copper and other metals using the antiport principle. Presently, this group of transporters is classified as superfamily 2.A.6 of the TCDB database [6]; this superfamily contains a great number of transporter proteins detected in all domains of life [7]. Within this superfamily, copper transporters belong to the HME (heavy metal efflux, 2.A.6.1) family.

Oxygenated aquatic ecosystems with high Cu^{2+} solubility and marine hydrotherms, where, due to the high temperature and high concentration of heavy

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metals, sufficient amounts of dissolved copper sulfides occur under anaerobic conditions, are regarded as possible habitats of microorganisms in which various copper detoxification mechanisms, including those based upon copper ATPases, could have emerged [1].

The recently described facultatively anaerobic, moderately thermophilic bacterium *Melioribacter roseus* [8] represents a suitable model for elucidating the relationship between the presence of the cuproproteome (the totality of copper-containing proteins) in an organism and its aerobic or anaerobic lifestyle. *Melioribacter roseus* gen. nov., sp. nov. [8] was characterized as a representative of the new family *Melioribacteraceae* within the new phylum *Ignavibacteriae*. This phylum belongs to the group of closely related phyla that also includes the phyla *Chlorobi* and *Bacteroidetes* [8, 9]. Apart from *M. roseus*, the phylum *Ignavibacteriae* includes one more culturable representative, *Ignavibacterium album* [10, 11]. Both organisms, *M. roseus* and *I. album*, are facultative anaerobes and organoheterotrophs [8]. According to the genomic and phenotypic characteristics of the representatives of *Chlorobi*, *Bacteroidetes*, and *Ignavibacteriae*, the following sequence of evolutionary events was suggested: (i) the separation of the common ancestral aerobic lineage into the branches of *Bacteroidetes* and the common ancestor of *Ignavibacteriae* and *Chlorobi* and (ii) the subsequent cleavage of the latter branch into *Ignavibacteriae* and *Chlorobi*, which was due to the new ecological niche occupied by *Chlorobi* and to the major changes in their metabolism [8].

M. roseus was isolated from a sample of the microbial biofilm that develops in the geothermal water outflow from the well 3P (Parabel District, Tomsk oblast), discharging deep subsurface aquifer from a depth of ca. 2700 m. Presumably, *M. roseus* migrated to the surface mat from the deep strata characterized by elevated concentrations of metals and metalloids [8]. Therefore, *M. roseus* should harbor a set of systems that control the intracellular content of toxic metals, including copper.

The goal of this work was to screen the genome of *M. roseus* for genes encoding copper proteins and copper homeostasis systems and to test the previously proposed hypothesis that the metabolism of *Ignavibacteriae* was primordially aerobic.

MATERIALS AND METHODS

Strain *Melioribacter roseus* P3M-2^T was from the culture collection of the Laboratory of Hyperthermophilic Microbial Communities of Winogradsky Institute of Microbiology, Russian Academy of Sciences. The resistance of the strain to Cu^{2+} ions was tested by cultivating it on the modified Widdel medium [8] supplemented with copper: sterile $CuCl_2$ solution was added to a final concentration of 10, 20, 30, or 50 mg/L.

The complete genome of *M. roseus* P3M-2^T, deposited at the NCBI GenBank (CP003557.1), was screened for the genes related to copper homeostasis. The target genes were identified from their similarity to the sequences specified for *Bacteria* and *Archaea* by Ridge et al. [1]. In addition, the conserved sequences of transmembrane α spiral IV of RND transporters [7] and the conserved domains of copper ATPases [12] were tested. The homologs of the genes detected in the *M. roseus* genome were found using the blastp algorithm with standard parameters [13]. The sets of *M. roseus* proteins and their retrieved homologs were used for phylogenetic analysis, performed with the MEGA5 software package [14]. The closest 100 homologs of each protein were chosen; thus, the total initial sequence array for the phylogenetic analysis consisted of 3 ATPases of *M. roseus* and 297 of their closest homologs. Using the CD-hit server [15], 100%-identical sequences (duplicates) were removed. 270 sequences remained, and the 3 incomplete sequences present among them (two *Firmicutes* sequences and one *Bacteroidetes* sequence) were deleted manually.The final sequence set comprised 267 sequences. The dendrograms were constructed using the Neighbor-Joining and Maximum Likelihood methods.

RESULTS

Genes encoding copper proteins are present in the *Melioribacter roseus* genome. In the *M. roseus* genome, several genes were revealed that encode various copper-containing proteins, including *cbb$_3$* (MROS_1513) and *cc(o/b)o$_3$* (MROS_0038) cytochrome *c* oxidases (oxygen reductases), an N_2O reductase (MROS_1106), and a laccase (MROS_0146) (Table 1). The closest homologs of these copper proteins were found to be encoded in the *I. album* genome. In particular, the identity level between the amino acid sequences of the copper-containing laccases of the two species, representing different families of the *Ignavibacteriae* phylum, was 52%. Laccase genes were identified in the genomes of many representatives of the *Chlorobi* phylum. Apart from *Chlorobi*, relatively close laccase amino acid sequences are present in representatives of the *Bacteroidetes* phylum, in *Caldithrix abyssi* (one of the two cultured representatives of the phylogenetically deep bacterial lineage close to *Chlorobi-Bacteroidetes-Ignavibacteriae*), and in *Firmicutes* representatives (the identity of laccase sequences of all the above mentioned groups to *M. roseus* laccases is 30-37%).

Five representatives of the phylum *Chlorobi* possess the genes of the copper-containing subunits of the *cbb$_3$* type cytochrome *c* oxidases. However, the corresponding gene clusters are incomplete in all of the five species. *Chlorobium chlorochromatii* lacks the structural subunit IV gene, whereas *C. phaeobacteroides,*

Table 1. The presence of copper protein genes in the genomes of *Ignavibacteriae*, *Chlorobi*, and *Bacteroidetes* representatives (according to the results of the analysis of the NCBI database)

	Ignavibacteriae		*Chlorobi*	*Bacteroidetes*
	M. roseus	*I. album*		
Enzymes of the cytochrome c oxidase family	+	+	–*	+
Copper nitrous oxide reductase	+	+	–	+
Copper oxidoreductase, polyphenol oxidase, laccase	+	+	+	+

* Except for *Chlorobaculum parvum*.

Table 2. Domain structure and the main conserved motifs of the functional domains of P type copper ATPases in *Meliribacter roseus* P3M-2

ATPase ORF number in the genome	N-terminal domain			Catalytic cytoplasmic domain		
	metal-binding motifs	stabilizing motif	heavy metals-binding membrane site	phosphorylation motif	phosphate-binding motif	motif of the "hinge" region which links the catalytic and the C-terminal domain
MROS_0327	CASC	**TGE**S	**CPC**	**DKTG**TIT	LL**TGD**	**VA**MV**GDG**I**ND**A**PAL**AQA**D**LSI**AI**
MROS_0791	CSTC...CASC	**TGE**P	**CPC**	**DKTG**TIT	**MITGD**	**VA**MV**GDG**I**ND**A**PAL**AQA**DIG**LA**I**
MROS_1511	CFHC... CNGC...CSSC	**TGE**S	**CPC**	**DKTG**TIT	LL**TGD**	**V**MMI**GDG**L**ND**AG**AL**QKS**D**

Note: Bold type marks amino acid residues that are the most conserved according to Chan et al. [16].

C. limicola, Chlorobaculum parvum, and *Pelodictyon phaeoclathratiforme* lack, in addition, the genes of the functional subunit III. *Chlorobium ferrooxidans* only possesses the gene of the copper subunit, which is equidistant from all of the known types of copper-containing oxygen reductases.

Homologs of the $cc(o/b)o_3$ type copper-containing cytochrome *c* oxidase are present in the genomes of *Bacteroidetes* and of one of the *Chlorobi* species, *Chlorobaculum parvum*. However, the gene cluster of this oxidase in *Bacteroidetes* differs in terms of its organization from the gene cluster of *M. roseus*. In *Chlorobaculum parvum*, it lacks the genes of two subunits (II and IV) of the enzyme.

***M. roseus* possesses several copper transport systems.** Cultivating of *M. roseus* at elevated Cu(II) concentrations revealed that the maximum copper concentration in the medium at which growth still occurred was 30 mg/L. The growth of *M. roseus* was completely inhibited by 50 mg/L Cu^{2+} in the culture medium.

Three copper ATPases were detected in the *M. roseus* genome. They all have four pairs of transmembrane helices characteristic of heavy metal ATPases (families 3.A.3.5 and 3.A.3.6) and exhibit a domain organization that is similar to that of these ATPases (Table 2). This organization implies the presence of metal-binding sites, the stabilizing TGEA motif of the cytosol loop in the N-terminal domain, conserved phosphorylation and phosphate-binding sites, and a 'hinge' region in the catalytic domain. The most variable domain is the third, N-terminal membrane domain [16].

Proteins IALB_0720 and IALB_2222 from *I. album* are among the closest homologs of the MROS_1511 and MROS_0327 ATPases; nonetheless, the *I. album* genome lacks a close homolog of MROS_0791 (Fig. 1). The MROS_1511 ATPase belongs to the operon encoding a cbb_3 cytochrome *c* oxidase (MROS_1513-1515) and its accessory proteins (Fig. 2c). The MROS_0327 ATPase comprises an operon (Fig. 2a) together with copper chaperone MROS_0326 and a transporter of the MFS family (2.A.1 according to TCDB), MROS_0328. Each of the neighboring genes of MROS_0791 and MROS_0790 ATPases contains one cupredoxin-like domain.

Apart from the genes encoding copper ATPases, the genome contains the genes of two putative copper transporters (MROS_0577 and MROS_0773) that transfer Cu^{2+} by proton antiport (the RND family, 2.A.6). The specific motifs of the conserved region of the transmembrane α helix IV (TMH IV) (Fig. 3) provide evidence that these proteins belong to the "RND-efflux" transporter group. The main function of RND transporters is the efflux of copper from the periplasmic space, as it was shown for the proteins encoded by *cusCFBA* operon in *E. coli* [17]. MROS_0481 and MROS_1253, the two other related proteins, lack characteristic motifs in the TMH IV region. Appar-

Fig. 1. Phylogenetic positions of the copper ATPases MROS_0327, MROS_0791, and MROS_1511 and related proteins. The dendrogram was constructed using the Neighbor Joining method and the Poisson distribution of amino acid substitution probability [23]. The Maximum Likelihood method and the Jones-Taylor-Thornton (JTT) model of amino acid substitution yielded a similar tree topology. Bar corresponds to substitution of 10% of amino acid residues. Numbers at the tree nodes are bootstrap values (100 iterations).

ently, these proteins belong to the HAE4 group of the HAE (hydrophobic and amphiphilic compounds efflux, 2.A.6.2, 2.A.6.5, 2.A.6.7) family of RND transporters transferring organic molecules [7].

Genes encoding all of the four transporters discussed above were included in the operons that also contained the genes of MFP (membrane fusion protein) and OMF (outer membrane factor) (Fig. 4). This allows prediction of localization of the transporters in the outer membrane. All proteins encoded by the operons that contain the MROS_0481 and MROS_0577 transporter genes displayed maximum similarity to those of *Calditrix abyssi*. Sequence identity between MROS_0481 and MROS_0577 and their

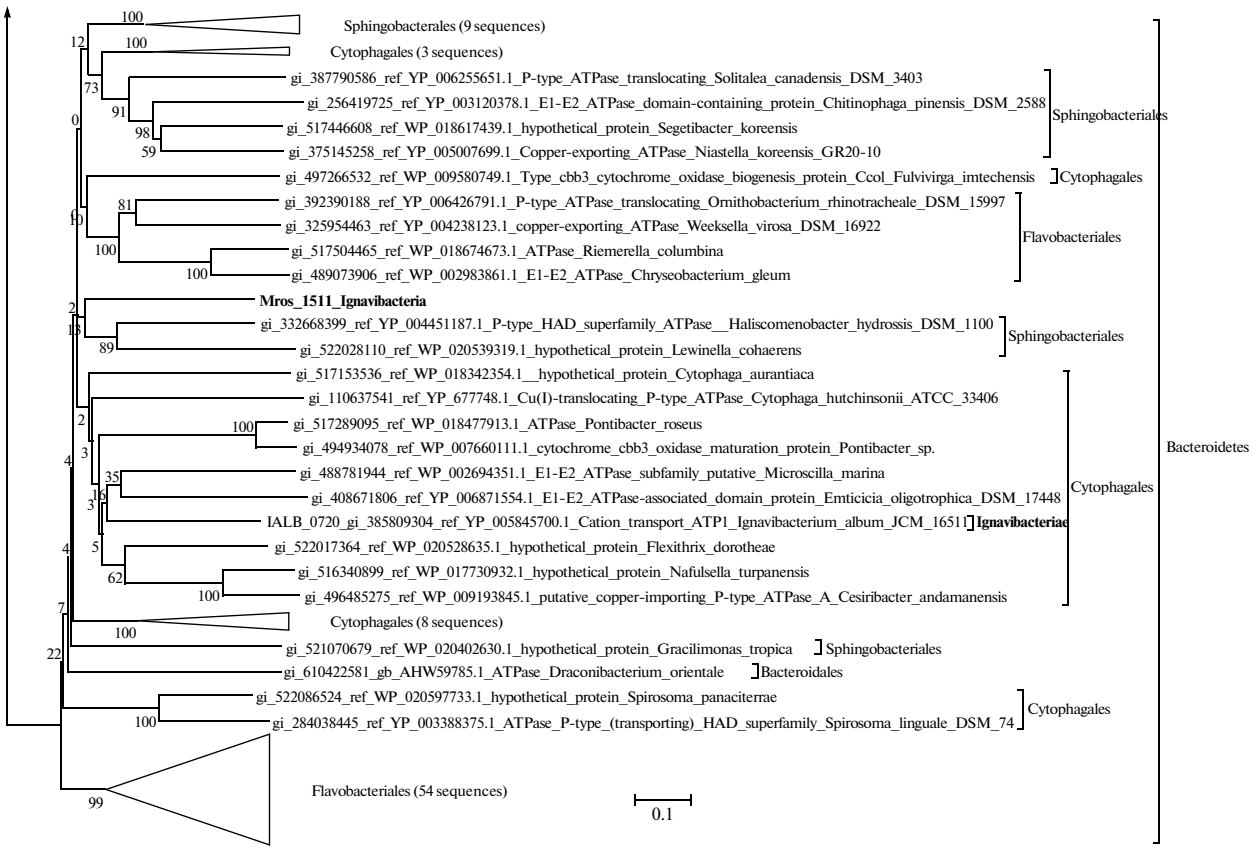

Fig. 1. (Contd.)

closest homologs was 58 and 72%, respectively. The closest homologs of all proteins encoded by MROS_0773- and MROS_1253-containing operons were *I. album* proteins; the identity level between the transporters per se and their closest relatives was 81 and 82%, respectively.

DISCUSSION

Our work demonstrated the presence of the genes encoding copper proteins in the genomes of two *Ignavibacteriae* representatives. All these genes have numerous homologs among representatives of *Bacteroidetes* (Table 1). Although a large number of *Chlorobi* species contain homologs of laccase genes, and some of them possess incomplete gene clusters of copper-containing cbb_3-type cytochrome *c* oxidases, only one of them, *Chlorobaculum parvum*, has a gene cluster of the copper-containing $cc(o/b)o_3$-type cytochrome *c* oxidase. However, it lacks the genes of two subunits of the enzyme, which allows suggesting the loss of the gene cluster of the $cc(o/b)o_3$-type oxygen reductase in representatives of *Chlorobi*. In *Ignavibacteriae* species this gene cluster possesses the same structure as in the representatives of the proteobacteria *Desulfovibriales* and *Desulfuromonadales* [8]. In *Desulfovibrio vulgaris*, the involvement of a $cc(o/b)o_3$ type cytochrome *c* oxidase in the generation of transmembrane potential was revealed [18]. Taking into account the high level of identity of the amino acid sequences of the copper-containing subunits of the oxidases of *M. roseus* and *I. album* with those of *D. vulgaris* (47% and 45%, respectively), it seems likely that this enzyme is involved in energy metabolism in *Ignavibacteriae* representatives.

Five representatives of the *Chlorobi* phylum (*Chlorobium phaeobacteroides, C. limicola, C. chlorochromatii, Chlorobaculum parvum*, and *Pelodictyon phaeoclathratiforme*) possess the genes of copper-containing cbb_3 type oxidoredutases. However, the gene cluster is incomplete in all of them. In addition, *C. ferrooxidans* contains one gene of the copper subunit of the oxygen reductase, equally distant from copper domains of cbb_3- and $cc(o/b)o_3$-type oxygen reductases. In compliance with the proposed hypothesis on the "secondary" anaerobiosis of *Chlorobi*, a possible evolutionary scenario is the loss of both accessory and catalytic copper-containing subunits by the organisms that have moved into a new anaerobic niche, while the bacteria that stayed in aerated environments retained the complete set of genes necessary for aerobic respiration.

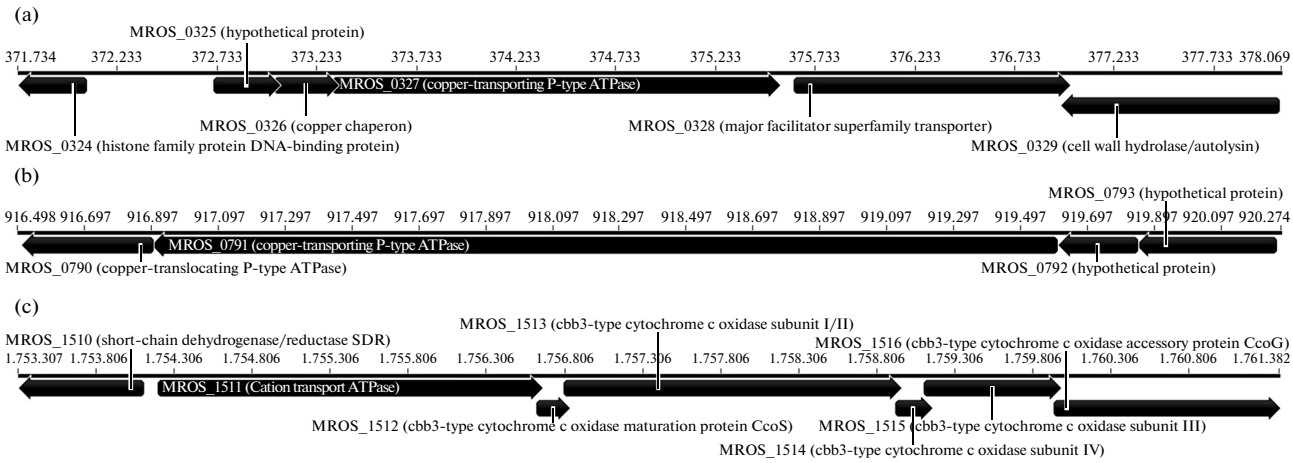

Fig. 2. Genomic context of the copper ATPases MROS_0327 (a), MROS_0791 (b), and MROS_1511 (c). The graphic image was obtained using Geneious software,version 4.8.5 (http://www.geneious.com).

1. AcrB (HAE5)	N	T	L	T	M	F	G	M	V	L	A	I	G	L	L	V	D	D	A	I	V	V	V	E	N
2. CzcA (HME1)	N	L	M	S	L	G	-	-	A	L	D	F	G	I	I	I	D	G	A	V	V	I	V	E	N
3. SilA (HME4)	N	I	M	S	L	G	G	I	A	I	A	V	G	A	M	V	D	A	A	V	V	M	I	E	N
4. CusA (HME4)	N	I	M	S	L	S	G	I	A	I	A	V	G	A	M	V	D	A	A	I	V	M	I	E	N
5. HP0969 (HME3b)	N	L	M	S	L	G	G	L	V	I	A	I	G	M	L	I	D	S	A	V	V	V	V	E	N
6. MROS_0773	N	I	M	S	L	G	G	I	A	I	A	I	G	A	M	V	D	A	S	I	V	L	V	E	N
7. MROS_0577	N	L	M	S	F	G	G	L	A	I	A	I	G	M	M	V	D	G	S	I	V	L	V	E	N
8. Altr5294 (HAE4)	N	V	F	S	L	G	G	L	A	L	G	V	G	I	V	V	D	N	S	I	V	M	L	E	N
9. MROS_0481	N	I	I	S	M	A	G	L	A	L	A	V	G	M	L	V	D	N	S	I	V	V	L	E	N
10. MROS_1253	N	I	I	S	L	S	S	L	S	I	A	V	G	L	V	V	D	D	A	I	V	I	L	E	N

Fig. 3. Characteristic sequences (signatures) of region IV of the transmembrane α helix [7] of various types of RND transporters of *Melioribacter roseus* (MROS_0773, MROS_0577, MROS_0481, and MROS_1253). 1. Group 5 of RND transporters of hydrophobic and amphiphilic compounds (HAE5) in the example of AcrB of *E. coli*; 2. Group 1 of RND transporters of heavy metals (HME1) in the example of CzcA (cobalt–zinc–cadmium) of *Ralstonia metallidurans*; 3. Group 4 of RND transporters of heavy metals (HME4) in the example of SilA of *Salmonella enterica* sv. *typhimurium*; 4.Group 4 of RND transporters of heavy metals (HME4) in the example of CusA of *E. coli*; 5. Group HME3b of RND transporters of heavy metals in the example of HP0969 of *Helicobacter pylori*; 6. MROS_0773; 7. MROS_0577; 8. Group 4 of RND transporters of hydrophobic and amphiphilic compounds (HAE4) in the example of Alr5294 of *Nostoc* sp.; 9. MROS_0481; 10. MROS_1253.

It should be noted that normal functioning of the copper proteome requires that the organism possesses a ramified copper homeostasis system involved in processing of copper-containing enzymes, as well as in the control of intracellular copper content during growth in a medium with dissolved Cu^{2+} compounds. Copper readily forms complexes and is chelated by organic compounds. Therefore, the concentration of bio-accessible copper depends on the composition of the culture medium used in the experiments on copper resistance evaluation. For instance, the Cu(II) concentration in the solution decreased from 200 to 150 mg/L immediately after adding $CuSO_4$ to the freshwater Widdel medium with lactate in the experiments aimed at determining the maximum copper concentration allowing the growth of copper-tolerant *Desulfovibrio* isolates [19]. Hence, it is impossible to make reasonable comparisons of the tolerance threshold values obtained in different laboratories with different culture media. Overall, the maximum estimated Cu^{2+} concentration at which *M. roseus* can grow (30 mg/L) is of the same order of magnitude as the values determined for the copper-intolerant but copper oxidoreductase-containing representatives of sulfate-reducing bacteria of the genera *Desulfovibrio* and *Desulfotomaculum* [20] grown in the same freshwater Widdel medium. The same soluble copper concentration range (tens of milligrams per L) is characteristic of anaerobic environments of deep subsurface biosphere. The inhibitory effect of 50 mg/L Cu^{2+} on *M. roseus* growth suggests either (i) that the copper transporters identified in the *M. roseus* genome are characterized by a high affinity to copper and are active at very low copper concentrations or (ii) that they can perform

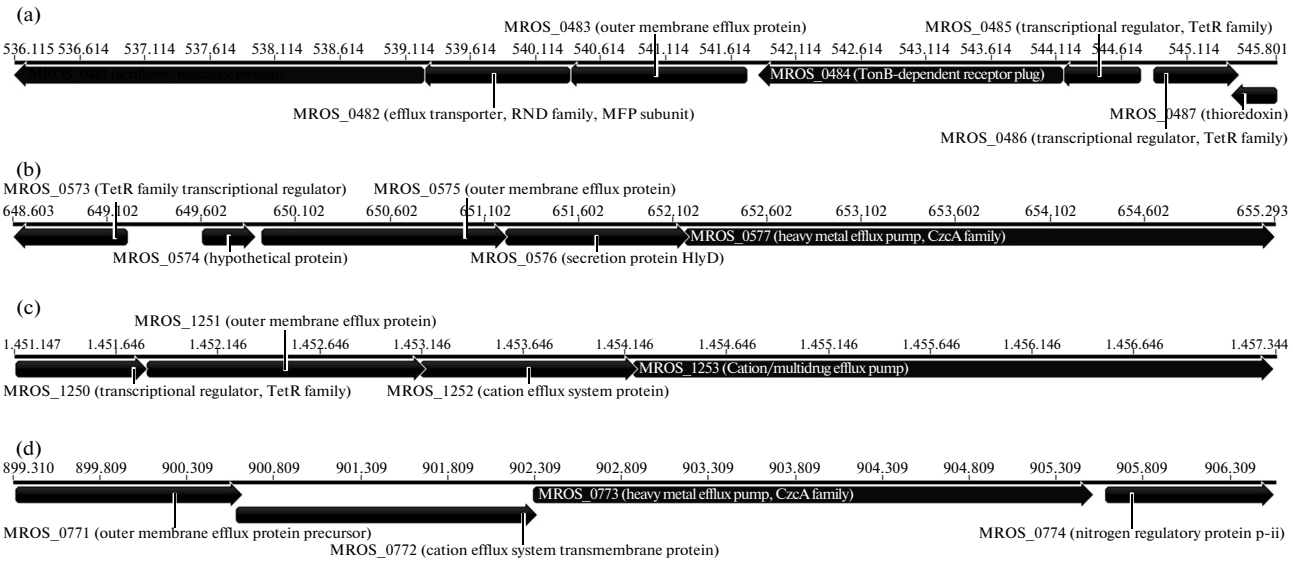

Fig. 4. Genomic context of the RND transporters MROS_0481 (a), MROS_0577 (b), MROS_1253 (c) and MROS_0773 (d). The graphic image was obtained using Geneious software,version 4.8.5 (http://www.geneious.com).

other functions apart from providing for copper tolerance, e.g. Cu^{2+} assimilation for the biosynthesis of copper-containing enzymes. Detailed analysis of the copper homeostasis systems identified in *M. roseus* is given below.

The main enzymes that transfer copper ions in/out of the cell are P-type copper-transporting ATPases (family 3.A.3 according to the TCDB database) [18], which have been revealed in all the three domains of life. In the *M. roseus* genome, three copper ATPases (copper-transporting ATPases) belonging to topological type I of P-type ATPases (family 3.A.3.5 according to TCDB) have been identified. The MROS_1511 copper ATPase is encoded in the operon with the genes of cbb_3 cytochrome *c* oxidase (MROS_1513-1515) and the genes related to its processing (Fig. 2c). This gives grounds for the suggestion that the MROS_1511 ATPase actually pumps Cu not out of the cytoplasm but in the opposite direction and, apart from the transport, it is possibly involved in assembling the cytochrome *c* oxidase. A number of gram-positive bacteria contain ATPases that transfer copper in different directions. A classic example is copper transfer in *Enterococcus hirae*, where the ATPase CopA is responsible for copper transfer into the cytoplasm and the ATPase CopB is responsible for excreting Cu into the periplasmic space. [3]. Phylogenetic analysis revealed that the MROS_1511 copper ATPase, together with IALB_0720, is located within a phylogenetic cluster of homologous proteins of *Bacteroidetes* representatives. However, these two ATPases belong to two remote branches of this cluster (Fig. 1). Both proteins apparently perform similar functions because their genes are located in operons that also contain cytochrome *c* oxidase genes. The close relationship of MROS_1511 and IALB_0720 with copper ATPases of different branches of *Bacteroidetes* probably results from independent horizontal transfer events of these genes in *M. roseus* and *I. album*. These transfers apparently occurred at a rather early evolution stage, before the split of *Bacteroidetes* phylum into four classes.

In contrast to MROS_1511, MROS_0791 is the only representative of *Ignavibacteriae* ATPases in a separated cluster of proteins. This points to the possibility that MROS_0791 could have been horizontally transferred to *M. roseus* (Fig. 1) or to its close ancestor after the separation from *I. album*, because the genome of the latter lacks close homologs of MROS_0791. The function of MROS_0791 is obviously related to the transfer of copper ions (supposedly, their excretion) and, presumably, silver ions. The domain structure of MROS_0791 is characteristic of a large number of known copper-transporting ATPases present in Pfam database. The neighboring gene MROS_0790 (Fig. 2c) contains a copper-binding cupredoxin-like domain and also seems to be involved in copper transport, although its exact role is yet unclear. Presumably, it acts analogously to the nonhomologous copper chaperone CopZ of the *cop* operon of *Enterococcus hirae* [2]. No MROS_0790 homologs have been identified in the *I. album* genome.

MROS_0327 and IALB_2222 constitute a clearly separated branch in the copper ATPases tree. The sequence belonging to a representative of *Bacteroidetes* is the homolog most closely related to this cluster. Along with the lack of other close homologs belonging to *Bacteroidetes*, this fact gives grounds for the suggestion that the MROS_0327 and IALB_2222 genes are vertically inherited. The genomic context of the ATPase MROS_0327—the copper chaperone

MROS_0326 and a transporter of the MFS family MROS_0328 (Fig. 2a)—is characteristic of detoxifying ATPases.

Thus, the phylogenetic analysis of the three copper ATPases of *M. roseus* and their genomic contexts indicate their different origins and different roles in the metabolism of the bacterium. It is highly probable that two of them were inherited horizontally, which is no surprise given the reported evidence that P-type ATPases are prone to horizontal transfer among prokaryotic organisms [18]. It seems likely that these ATPases were obtained from microorganisms belonging to *Firmicutes* and *Bacteroidetes*, widespread in *M. roseus* habitats, which facilitates horizontal gene transfer between them. The capacity of *M. roseus* to form biofilms is an additional factor that promotes the transfer [21]. Presumably, the MROS_0327 gene was vertically inherited from the common ancestor of *Ignavibacteriae, Chlorobi*, and *Bacteroidetes*. The lack of close MROS_0327 homologs in the representatives of the latter two phyla (except for one sequence in *Nafulsella turpanensis*, belonging to *Bacteroidetes*) suggests that they have lost these genes during the course of evolution. The retention of the putative operon of Cu^{2+} detoxification system in *M. roseus* emphasizes the importance of maintaining copper homeostasis in geothermal water ecosystem characterized by elevated heavy metal content. However, it should be noted that in *Bacteroidetes* a fairly large number of copper ATPases are known, which may result from their metabolic versatility, as well as may be due to the large number of sequenced genomes of the representatives of this phylum. At the same time, only 15 homologs of copper-transporting ATPases have been revealed so far in the 12 publicly available (as of November 21, 2014) genomes of *Chlorobi* representatives. All these homologs are phylogenetically distant from the copper ATPase genes of *Ignavibacteriae*. None of them contains genes of copper-containing cytochrome *c* oxidases in the genomic context, which confirms participation of these ATPases in detoxification (excretion) of copper ions but not in the processing of oxidoreductases. This correlates with the strictly anaerobic mode of metabolism of *Chlorobi*. The same function is performed by the RND-transporters revealed in both *Ignavibacteriae* and *Chlorobi* representatives.

In general, identification of copper oxidoreductases (laccases and *cc(o/b)o*$_3$- and *cbb*$_3$-type cytochrome *c* oxidases) and several copper homeostasis systems in *M. roseus*, as well as the results of their phylogenetic analysis allow an assumption to be made that the ancestral forms of *Ignavibacteriae* evolved in aerobic ecological niches containing soluble copper compounds. Thus, our data are consistent with the hypothesis about "aerobic roots" of the phylum *Ignavibacteriae*, supporting the idea that the common ancestor that gave rise to *Ignavibacteriae* and *Chlorobi* possessed aerobic mode of metabolism.

This evolutionary scenario implies the emergence of secondary anaerobiosis in the representatives of the phylum *Chlorobi*, which have drastically changed their metabolism to anaerobic upon occupation of a novel environmental niche in the course of evolution [22].

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