Recent Origin of the Methacrylate Redox System in Geobacter sulfurreducens AM-1 through Horizontal Gene Transfer

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Abstract

The origin and evolution of novel biochemical functions remains one of the key questions in molecular evolution. We study recently emerged methacrylate reductase function that is thought to have emerged in the last century and reported in Geobacter sulfurreducens strain AM-1. We report the sequence and study the evolution of the operon coding for the flavin-containing methacrylate reductase (Mrd) and tetraheme cytochrome (Mcc) in the genome of G. sulfurreducens AM-1. Different types of signal peptides in functionally interlinked proteins Mrd and Mcc suggest a possible complex mechanism of biogenesis for chromoproteins of the methacrylate redox system. The homologs of the Mrd and Mcc sequence found in δ-Proteobacteria and Deferrribacteres are also organized into an operon and their phylogenetic distribution suggested that these two genes tend to be horizontally transferred together. Specifically, the mrd and mcc genes from G. sulfurreducens AM-1 are not monophyletic with any of the homologs found in other Geobacter genomes. The acquisition of methacrylate reductase function by G. sulfurreducens AM-1 appears linked to a horizontal gene transfer event. However, the new function of the products of mrd and mcc may have evolved either prior or subsequent to their acquisition by G. sulfurreducens AM-1.

Introduction

Anaerobic bacteria frequently use unsaturated organic compounds as terminal electron acceptors [1]. Among such forms of respiration, fumarate respiration of anaerobes has been studied most extensively [2–8]. During fumarate respiration bacterial cells reduce fumarate in the cytosol (e.g. Wolinella succinogenes and Escherichia coli) or in the periplasm (as in Shewanella).
The cytosolic fumarate-reducing enzyme complex is located at the inner side of the cytoplasmic membrane and consists of 3 or 4 protein subunits [2–7]. Periplasmic fumarate reductases of the bacterial genus *Shewanella* are soluble monomers belonging to the flavocytochrome *c* family [9–16]. Data on enzyme systems and electron transport chain components that use other double-bond compounds as terminal electron acceptors are often fragmentary and contradictory or completely absent [1].

Anaerobic bacterium *Geobacter sulfurreducens* AM-1 was isolated in the study of decomposition of methacrylate industry waste [17]. The *G. sulfurreducens* AM-1 strain is capable of complete oxidation of acetate coupled to reduction of methacrylate (2-methylpropenoate), an anthropogenic compound that serves as the terminal acceptor of the bacterial reductase chain [18]. The study of *Geobacter* species (Deltaproteobacteria) is of applied interest due to their significant role in bioremediation of radioactive metals [19–22]. They serve as important agents in the global cycles of metals and carbon, reducing Fe(III) to Fe(II) and U(VI) to U(IV), oxidizing acetate and other organic compounds and participating in humus decomposition. Furthermore, they are fumarate-respiring organisms [19–21,23] and electrotrophs [24].

Transformation of methacrylate to isobutyrate occurs in the periplasm of bacterium *G. sulfurreducens* AM-1 [25] by the periplasmic flavin-containing methacrylate reductase Mrd (50 kDa) [1,18]. Mrd activity depends on periplasmic tetraheme cytochrome *c* Mcc (30 kDa), which is the physiological electron donor for this enzyme. Furthermore, the two-component methacrylate redox system catalyzes reduction of acrylate, which is a compound found in nature [26], at a rate comparable to that for synthetic methacrylate, while lacking fumarate reduction [18].

Membranes of bacterium *G. sulfurreducens* AM-1 contain menaquinone-8 (menaquinone with 8 isoprene residues in the side chain), which transfers reducing equivalents to the methacrylate redox system from the citric acid cycle [1,18]. The electron carrier from menaquinone to Mcc remains unknown, although the periplasmic cytochromes *c* (12.5 and 15.5 kDa) and the membrane cytochrome *c* (67.6 kDa) are possible candidates [27].

N-terminal amino acid sequences, 27 and 29 amino acids in length, respectively, were identified from purified Mrd and Mcc [18]. Previous analysis suggested that the Mrd sequence was homologous to flavocytochromes *c* in several bacterial and a few archaeal genomes [28]. However, the length of the Mrd fragment was not long enough to perform a comprehensive sequence analysis of the two proteins that have recently evolved into the methacrylate redox system. Furthermore, the Mcc amino acid sequence has not been investigated.

Methacrylate, a common monomer in polymer plastics and resins, is strictly a man-made molecule [29]. It is also the main substrate of the methacrylate redox system and, therefore, methacrylate-based respiration might have evolved sometime in the second half of the 20th century. The *G. sulfurreducens* AM-1 strain is the only known strain capable of methacrylate respiration [1,17] and, therefore, the sequences of the methacrylate redox system genes provide an unparalleled opportunity to study the evolutionary history of a novel system of respiration.

Here we report the sequence of the two genes of the methacrylate redox system from the *G. sulfurreducens* AM-1 genome, analyze their translation products and study their evolutionary origins.

**Results**

**Organization of the mrd and mcc genes in the Geobacter sulfurreducens AM-1 genome**

We sequenced the genome of *G. sulfurreducens* AM-1, obtaining a draft with a single contig. To localize the *mrd* and *mcc* genes, we mapped the previously identified short 27 and 29 amino
acid sequences [18] to the genome sequence. We found that the genes coding for Mrd and Mcc were arranged linearly and organized in one transcription unit (Fig 1). The mrd gene (1581 bp) was separated by 56 nucleotides from mcc (696 bp). The genes were flanked by a transposase gene 3297 nucleotides upstream of mrd separated from mrd by two pseudogenes and GTP cyclohydrolase gene 505 nucleotides downstream of mcc. Both flanking genes have the same orientation as mrd and mcc.

Putative promoter sites were found in close proximity to the predicted start codon. The sequences found 75 to 97 bp upstream of the translation start codon are similar to the consensus promoter sequences typically found -10 to -35 from the transcription start site. Furthermore, two transcription factor binding sites are predicted in this region, supporting the hypothesis that the promoter is a common regulatory element of the redox operon. A potential ρ-independent transcriptional terminator (energy of terminator -8.9) was found 75 nucleotides downstream of mcc. A second potential transcriptional terminator (terminator energy -9.4) is located in the spacer between the two genes and partially overlapped the mcc gene. The extra transcription termination signal located between the genes in the operon implies a complex regulation of the redox system at the transcriptional level.

Evolution of the methacrylate redox system

To elucidate the evolutionary history of the methacrylate redox system, we searched for orthologues of mrd and mcc. First, we searched for homologs in the eleven Geobacter genomes available in GenBank. For mrd the closest homologs by protein sequence divergence were found in three strains: G. lovleyi SZ (YP_001951186.1, YP_001953845.1, YP_001953762.1), G. bemidjiensis Bem (YP_002140822.1, YP_002140385.1) and Geobacter sp. M21 (YP_003023900.1) (Table 1, Fig 2a). One of the homologs from G. lovleyi SZ, capable of chlororespiration, was the only protein from this list (YP_001951186.1) that does not contain the heme-binding sites CXXCH. Other homologous sequences found in Geobacter genomes have 4 heme-binding sites and different regions of their sequence are homologous to either Mrd or Mcc from the methacrylate redox system of G. sulfurreducens AM-1 (Tables 1 and 2; Fig 2a and 2b). Homology of Mcc from G. sulfurreducens AM-1 was observed for N-terminal amino acid sequence of Geobacter species flavocytochromes (usually 125 amino acids from the N-terminus). Sequence identity of Mrd with the flavocytochromes was higher (see column 5 of Tables 1 and 2) than for the region homologous to Mcc, and found in the C-terminal region (usually between the
| Class          | Species                          | GenBank accession number | Annotated function                  | % Similarity / Identity | Length of alignment with Mrd, e-value | Length, calculated Mr (kDa) of immature protein | Type of cleavable signal peptide (length) | Tat-motif | Heme-binding sites |
|---------------|----------------------------------|--------------------------|------------------------------------|-------------------------|--------------------------------------|---------------------------------------------|---------------------------------------|-----------|-----------------|
| Δ-proteobacteria | Geobacter sulfurreducens AM-1 | methacrylate reductase | 100/100                           | 526 aa 57.2 kDa         | Tat (55 aa) RRDFLK no                |                                              |                                       |           |                 |
| Δ-proteobacteria | Anaeromyxobacter sp. K | flavocytochrome c | 78/64 96, 0.0                       | 515 aa 55.5 kDa         | Tat (38 aa) RRAMLK no                |                                              |                                       |           |                 |
| Δ-proteobacteria | Anaeromyxobacter dehalogenans 2CP-1 | flavocytochrome c | 78/64 96, 0.0                       | 515 aa 55.6 kDa         | Tat (38 aa) RRAMLK no                |                                              |                                       |           |                 |
| Δ-proteobacteria | Anaeromyxobacter dehalogenans 2CP-C | flavocytochrome c | 78/62 95, 0.0                       | 511 aa 54.9 kDa         | Tat (42 aa) RRSVIK no                |                                              |                                       |           |                 |
| Δ-proteobacteria | Desulfatibacterium alkenivorans AK-01 | flavocytochrome c | 78/65 95, 0.0                       | 507 aa 55.0 kDa         | Tat (40 aa) RRGLLQ no                |                                              |                                       |           |                 |
| Δ-proteobacteria | Geobacter lovleyi SZ | flavocytochrome c | 53/39 96, 8e-97                     | 517 aa 56.0 kDa         | Tat (43 aa) RRSLFK no                |                                              |                                       |           |                 |
| Δ-proteobacteria | Geobacter lovleyi SZ | flavocytochrome c | 54/39 87, 2e-71                     | 596 aa 63.3 kDa         | Tat (26 aa) Sec (25 aa) no           |                                              |                                       |           |                 |
| Δ-proteobacteria | Geobacter lovleyi SZ | flavocytochrome c | 51/38 95, 5e-72                     | 589 aa 61.7 kDa         | Tat (26 aa) Sec (25 aa) no           |                                              |                                       |           |                 |
| Δ-proteobacteria | Geobacter bemidjiensis Bem | flavocytochrome c | 55/40 88, 1e-78                     | 598 aa 63.3 kDa         | Tat (25 aa) Sec (25 aa) no           |                                              |                                       |           |                 |
| Δ-proteobacteria | Geobacter bemidjiensis Bem | flavocytochrome c | 51/38 88, 5e-72                     | 591 aa 61.5 kDa         | Tat (21 aa) Sec (25 aa) no           |                                              |                                       |           |                 |
| Δ-proteobacteria | Geobacter sp. M21 | flavocytochrome c | 55/40 88, 1e-78                     | 598 aa 63.2 kDa         | Tat (25 aa) Sec (25 aa) no           |                                              |                                       |           |                 |
| Δ-proteobacteria | Shewanella frigidimarina NCIMB 400 | flavocytochrome c | 55/38 95, 8e-91                     | 510 aa 54.9 kDa         | Tat (35 aa) RRHFLK no                |                                              |                                       |           |                 |
| Δ-proteobacteria | Shewanella frigidimarina NCIMB 400 | flavocytochrome c | 53/36 86, 3e-76                     | 588 aa 63 kDa           | Tat (22 aa) Sec (25 aa) no           |                                              |                                       |           |                 |
| Δ-proteobacteria | Shewanella frigidimarina NCIMB 400 | flavocytochrome c | 50/34 93, 1e-64                     | 507 aa 53.8 kDa         | Tat (34 aa) RRNIK no                 |                                              |                                       |           |                 |
| Δ-proteobacteria | Shewanella onedinensis MR-1 | periplasmic fumarate reductase FccA | 54/38 86, 2e-72 | 596 aa 62.0 kDa | Sec (25 aa) no | | | | |
| Δ-proteobacteria | Shewanella onedinensis MR-1 | urocanate reductase SO_4620 | 39/36 89, 2e-83 | 582 aa 62.2 kDa | Sec (30 aa) no | | | | |
| Δ-proteobacteria | Shewanella frigidimarina NCIMB 400 | Periplasmic fumarate reductase; flavocytochrome c | 51/35 84, 2e-63 | 596 aa 63.0 kDa | Sec (25 aa) no | | | | |

(Continued)
140th and 590th amino acids). Thus, the methacrylate redox system homologs of bacteria of the genus *Geobacter* are often present as one multifunctional flavoprotein, combining functions of electron delivery and catalysis of reduction.

A diversity of other cytochrome *c* protein sequences were found to be coded in *Geobacter* genomes [30–36], which were much more diverged than the *Geobacter* homologs we considered in our phylogenetic analysis. None of these distantly related genes were considered in our analysis.

Homologs of both Mrd and Mcc with higher sequence identity were found outside the *Geobacter* genus in a few species with a broad phylogenetic distribution, indicating a complex evolutionary origin of these proteins in *G. sulfurreducens* AM-1. The distribution of Mrd homologs varied across bacterial clades. The closest of the identifiable Mrd homologs (78% similarity; Table 1, Fig 2a), which were annotated as flavoproteins, were from δ-Proteobacteria: *Anaeromyxobacter dehalogenans* 2CP-C (YP_465303.1), *A. dehalogenans* 2CP-1 (YP_002492269.1), *A. sp. K* (YP_002134140.1) and *Desulfatibacillum alkenivorans* AK-01 (YP_002429921.1) and *Deferribacteres: Denitrovibrio acetiphilus* DSM 12809 (YP_003505239.1).

**Table 1.** (Continued)

| Class            | Species                        | GenBank accession number | Annotated function | % Similarity / Identity | Length of alignment with Mrd, e-value | Length, calculated Mr (kDa) of immature protein | Type of cleavable signal peptide (length) | Tat-motif hemebinding sites CXXCH |
|------------------|--------------------------------|--------------------------|--------------------|-------------------------|---------------------------------------|-----------------------------------------------|-------------------------------------------|---------------------------------|
| E- Proteobacteria| *Wolinella succinogenes* DSM 1740 | NP_9063881.1*            | flavocytochrome *c* flavin subunit FccA | 51/35                   | 87, 2e-67                             | 515 aa 55.9 kDa                                | Tat (34 aa) RRDLIK no               |

* The last three proteins in the table have lower sequence similarity with methacrylate reductase. They were included in the table as they have been characterized biochemically.

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**Fig 2.** Phylogeny reconstructions for Mrd (A) and Mcc (B) homologs. Unrooted trees are shown with posterior probabilities. Unlabeled nodes have a posterior probability of 1. The following sequences were used. A (Mrd): *D. alkenivorans* AK-01 (YP_002429921.1), *D. acetiphilus* DSM 12809 (YP_003505239.1), *A. dehalogenans* 2CP-1 (YP_002492269.1), *A. dehalogenans* 2CP-C (YP_465303.1), *S. marimammalium* (WP_018370472.1), *S. frigidimarina* NCIMB 400 (YP_751265.1), *G. lovleyi* SZ (YP_001953762), *G. bemidjiensis* Bern (YP_002140385), *G. lovleyi* SZ (YP_001953845), *G. sp. M21*, *G. bemidjiensis* Bern (YP_002140022) B (Mcc): *P. excrementihominis* YIT 11859 (WP_008864032.1), *D. alkenivorans* AK-01 (YP_002429920.1), *D. acetiphilus* DSM 12809 (YP_003505238.1), *A. dehalogenans* 2CP-C (YP_465304.1), *G. bemidjiensis* Bern (YP_002140822.1, YP_002140385.1), *G. lovleyi* SZ (YP_001953845.1), *G. sp. M18* (YP_004300524.1), *G. sp. M21* (YP_003023900.1).

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| Class       | Species                          | GenBank accession number | Annotated function | % Similarity / Identity | Length of alignment with Mcc, e-value | Length, calculated Mr (kDa) of immature protein | Type of cleavable signal peptide (length) | Heme-binding sites |
|------------|----------------------------------|-------------------------|-------------------|-------------------------|--------------------------------------|-----------------------------------------------|------------------------------------------|------------------|
| Δ- Proteobacteria | Geobacter sulfurreducens AM-1 | YP_002134139.1          | cytochrome c       | 100/100                 | 84, 2e-40                           | 221 aa 22.9 kDa                                | Sec (23 aa)                              |                  |
| Δ- Proteobacteria | Geobacter sulfurreducens AM-1 | YP_002492268.1          | hypothetical protein A2cp1_1860 | 56/42                  | 85, 2e-39                           | 221 aa 22.9 kDa                                | Sec (24 aa)                              |                  |
| Δ- Proteobacteria | Anaeromyxobacter dehalogenans 2CP-C | YP_465304.1*          | hypothetical protein Adeh_2097 | 56/42                  | 84, 3e-47                           | 233 aa 24.1 kDa                                | Sec (24 aa)                              |                  |
| Δ- Proteobacteria | Desulfatibacillum alkenivorans AK-01 | YP_002429920.1        | hypothetical protein Dalk_0747 | 51/40                  | 44, 2e-15                           | 109 aa 12.1 kDa                                | no                                        |                  |
| Deferribacteres | Denitrovibrio acetiphilus DSM 12809 | YP_003505238.1*      | hypothetical protein Dacet_2522 | 48/36                  | 94, 1e-32                           | 208 aa 22.9 kDa                                | Sec (18 aa)                              |                  |
| Δ- Proteobacteria | Geobacter lovleyi SZ | YP_001953845.1          | flavocytochrome c   | 54/44                   | 42, 1e-13                           | 596 aa 63.3 kDa                                | Sec (25 aa)                              |                  |
| Δ- Proteobacteria | Geobacter lovleyi SZ | YP_001953762.1          | flavocytochrome c   | 50/39                   | 42, 4e-10                           | 589 aa 61.7 kDa                                | Sec (26 aa)                              |                  |
| Δ- Proteobacteria | Geobacter bemidjensis Bem | YP_002140822.1          | flavocytochrome c   | 48/42                   | 41, 2e-11                           | 598 aa 63.3 kDa                                | Sec (25 aa)                              |                  |
| Δ- Proteobacteria | Geobacter bemidjensis Bem | YP_002140385.1          | flavocytochrome c   | 59/44                   | 35, 1e-12                           | 591 aa 61.5 kDa                                | Sec (21– 27 aa)                          |                  |
| Δ- Proteobacteria | Geobacter sp. M18 | YP_004200524.1          | flavocytochrome c   | 62/51                   | 35, 3e-14                           | 584 aa 60.7 kDa                                | Sec (22– 23 aa)                          |                  |
| Δ- Proteobacteria | Geobacter sp. M21 | YP_003023900.1          | flavocytochrome c   | 48/42                   | 41, 7e-11                           | 598 aa 63.2 kDa                                | Sec (25 aa)                              |                  |
| Γ- Proteobacteria | Shewanella frigidimarina NCIMB 400 | Q07WU7.2 (FccD)       | fumarate reductase flavoprotein subunit; flavocytochrome c | 53/38                  | 48, 6e-11                           | 596 aa 63.0 kDa                                | Sec (25 aa)                              |                  |
| Γ- Proteobacteria | Shewanella frigidimarina NCIMB 400 | YP_751265.1* (IflcD) | flavocytochrome c   | 51/39                   | 40,1e-09                           | 588 aa 63 kDa                                  | Sec (22 aa)                              |                  |
| Γ- Proteobacteria | Shewanella frigidimarina NCIMB 400 | YP_751191.1          | tetraheme flavocytochrome c | 50/34                  | 41, 6e-07                           | 122 aa 13.9 kDa                                | Sec (22 aa)                              |                  |
| Γ- Proteobacteria | Shewanella oneidensis MR-1 | NP_716599.1 (FccD)     | periplasmic fumarate reductase FccA | 59/46                  | 35, 3e-10                           | 596 aa 62.4 kDa                                | Sec (25 aa)                              |                  |
| B- Proteobacteria | Parascuaternera excrementihominis YIT 11859 | WP_0008864032.1      | hypothetical protein HMREF9439_01147 | 50/33                  | 80, 7e-25                           | 208 aa 22.7 kDa                                | Sec (19 aa)                              |                  |
| E- Proteobacteria | Wolinella succinogenes DSM 1740 | NP_906387.1**             | flavocytochrome c heme subunit | 44/28                 | 51, 0.002                           | 146 aa 16.6 kDa                                | Sec (26 aa)                              |                  |

*Annotated by us.

**The last protein in the table has lower sequence similarity than others. It was included because it has been characterized biochemically.

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Interestingly, the same species that harbor the closest homologs of Mrd also have the closest homologs of Mcc (48–58% similarity; Table 2, Fig 2b): *A. dehalogenans* 2CP-C (YP_465304.1), *A. dehalogenans* 2CP-1 (YP_002492268.1), *A. sp. K* (YP_002134139.1), *D. alkenivorans* AK-01 (YP_002429920.1), *D. acetiphilus* DSM 12809 (YP_003505238.1). We annotated them as multiheme cytochrome c (Table 2). The redox systems of these organisms were represented by two proteins with their genes organized in one transcriptional unit. The only exception to having a close homologue of both Mdr and Mcc was *Parasutterella excrementihominis* YIT 11859 (β-Proteobacterium) that had a close homolog only of Mcc (WP_008864032.1, Table 2, Fig 2b).

To confirm that the *G. sulfurreducens* AM-1 Mrd and Mcc homologs found in other *Geobacter* species are not their direct orthologues, we performed a phylogenetic analysis of the homologs, including several of the sequences from the *Geobacter* genus that were most similar to Mrd of *G. sulfurreducens* AM-1. The analysis showed that *G. sulfurreducens* AM-1 Mrd and Mcc share a closer common ancestor with sequences from distant clades of bacteria, confirming that the methacrylate redox system genes, *mrd* and *mcc*, were likely acquired by *G. sulfurreducens* AM-1 through recent horizontal gene transfer and that their orthologues are not present in the sequenced *Geobacter* genomes (Fig 2a and 2b).

**Products of the methacrylate redox system genes**

The protein coded by *mrd* has 526 amino acids (Mr 57.2 kDa). The N-terminal amino acid sequence contains a 55 amino acid-long signal peptide with the Tat-motif RRDFLK in position 25 (Fig 3, Table 1). Thus, the mature protein is predicted to contain 471 amino acids (estimated Mr 51.4 kDa). Previous results have shown that the mature Mrd has 1 mol FAD [18]; therefore, the Mr of the mature Mrd with FAD should be 52.2 kDa, which is consistent with experimental data. We validated the start and flanking regions of *mrd* by Sanger sequencing of both strands, which were identical to the sequences obtained through the next generation sequencing of the entire genome. Thus, the unusually long predicted signal peptide was confirmed not to result from sequencing or assembly error.

The *mcc* gene codes for a protein 231 amino acids long (Mr 24.5 kDa). The N-terminal region contains a shorter Sec-type signal peptide of 23 amino acids (Fig 4, Table 2) with the mature protein predicted to have 208 amino acids (Mr 22.1 kDa). Previous experiments showed that the mature Mcc had 4 mol of heme c and a Mr of nearly 30 kDa [16]. Consistent with these results, we found four heme-binding motifs CXXCH [37] with the GENE RUNNER program. The Mr of a mature Mcc with 4 hemes is 24.8 kDa, substantially lower than expected. A visual analysis of the Mcc sequence revealed three more heme-binding motifs CXXCH, which brought the Mr of the mature Mcc with 7 hemes to 27.9 kDa (Fig 4).

The closest of the identifiable homologs of Mrd (Fig 2a) are likely FAD-binding proteins and flavocytochromes c, as indicated by the conserved phosphate-binding regions of N-termini (Fig 3). The phosphate-binding site is typical for all FAD- and NAD(P)H-dependent oxidoreductases: xhxhGxGxxGxxxxxhx(x),hxxhE(D), where x — any amino acid, h — hydrophobic amino acid [38]. In the case of Mrd this site was located between amino acids 69 and 98 of the immature protein (Fig 3). The central part of the consensus, GxGxxG, is a glycine-rich part of the loop, linking the first β-sheet in the Rossmann fold with the first α-helix directed to the pyrophosphate residue for charge compensation. Generally this motif has β-strand-turn-β-strand structure and forms a flexible clamp, surrounding and anchoring the pyrophosphate of FAD [39]. Another conservative FAD-binding site, which is an eleven amino acid segment T(S)xxxxxF(Y)hhGD(E) [40], was present in amino acid sequences of Mrd and its homologs. The site was slightly truncated, without the first threonine while all other amino acids were present (487–491).
The heme-binding sites of Mcc homologs identified by the phylogenetic analysis (Fig 2b) are shown in Fig 4. Their presence suggests that these homologs, not annotated as having any function, are cytochromes c, containing four heme-binding sites (YP_002429920.1) in *D. alkenivorans* AK-01 or seven heme-binding sites in the other species.

The Mrd sequence of *G. sulfurreducens* AM-1 had a higher level of similarity with its homologs (Table 1) than Mcc of *G. sulfurreducens* AM-1 (Table 2). This observation is consistent with a relatively poor conservation of cytochromes c[36] and probably with evolutionary early origin of the flavin-containing Mrd homologs.

**Fig 3.** Multiple protein sequence alignment of Mrd coded in *Geobacter sulfurreducens* AM-1 and its closest flavocytochrome c homologs from *Desulfatibacillum alkenivorans* AK-01 (YP_002429921.1); *Denitrovibrio acetiphilus* DSM 12809 (YP_003505239.1) and *Anaeromyxobacter dehalogenans* 2CP-1 (YP_002492269.1).

Amino acid sequences of Mrd homologs (YP_002134140.1, YP_002492269.1, YP_465303.1) of all three mentioned representatives of the genus *Anaeromyxobacter* are very similar. Therefore, we used the sequences of the Mrd homolog only from *A. dehalogenans* 2CP-1 (YP_002492269.1) as one representative of the genus. Cleavable signal peptides of Tat type are underlined; the Tat motif is shown in bold. Conserved pyrophosphate-binding sites and amino acids presumably involved in catalysis are highlighted in green. Probable proton donor is marked in red.

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The Mrd sequence of *G. sulfurreducens* AM-1 had a higher level of similarity with its homologs (Table 1) than Mcc of *G. sulfurreducens* AM-1 (Table 2). This observation is consistent with a relatively poor conservation of cytochromes c[36] and probably with evolutionary early origin of the flavin-containing Mrd homologs.
Discussion

The methacrylate redox system genes in the genome of *G. sulfurreducens* AM-1 appear to be arranged in a single operon. The clear absence of orthologs in the genomes of several other *Geobacter* genomes, coupled with a lack of closely-related orthologs in genomes of bacteria from any other closely related genus, strongly suggests that the methacrylate redox system genes were acquired recently by the *G. sulfurreducens* AM-1 strain (Fig 5). The intriguing similarity of the phylogenetic distribution of the closely related homologs of both genes, *mrd* and *mcc*, suggests that these two genes tend to be horizontally transferred together, confirming their close functional relationship. The high congruence of the evolutionary history of the *mrd* and *mcc* genes is consistent with their organization into a single operon and confirms their joint functional role.

Unfortunately, for most of the identified homologs experimental data of their enzyme specificity are not available. Such lack of experimental data precludes us from understanding whether or not the acquisition of the methacrylate reducing function occurred before or after the horizontal gene transfer. Furthermore, even the closest of the identified homologs were evidently too diverged to be identified as the origin of the horizontal gene transfer. This conclusion is based on the observation of the divergence of Mrd and Mcc sequences from their closest homologs in comparison to the high similarity of genomes of different *Geobacter* species. Nevertheless, some experimentally characterized proteins can be distinguished among the homologs of the methacrylate redox system. The characterized homologs of Mrd include flavo-protein FccA (NP_906388.1) from *Wolinella succinogenes* [41], an urocanate reductase *SO_4620* (NP_720136.1; [42]) and periplasmic fumarate reductases Fcc3 (Q07WU7.2; [9, 10, 43]), I fc3 (YP_751265.1; [11,12]) and Fcc3 (NP_716599.1; [13–15]) from bacteria of the genus *Shewanella* (Table 1). *Shewanella’s* periplasmic fumarate reductases are cytochromes *c* homologs as well (Table 2). Therefore, the methacrylate redox system and its homologs reduce...
the double bonds of unsaturated organic compounds (such as acrylate, methacrylate, urocar-
ate, fumarate), using them as terminal acceptors of reducing equivalents. None of the species
or strains described previously are known to grow by respiration of methacrylate.

Conserved amino acids (histidine-461 and arginines—R501 and R353, Fig 3), found in the
Mrd sequence, may stabilize the transition state during catalysis by providing delocalization of
the negative charge of the intermediate carbanion, in a similar manner as in Shewanella fuma-
rate reductases [16]. Point mutagenesis showed that the arginine homologous to R353 of Mrd
is the proton donor for the carbanion [44]. The fumarate reductase arginine homologous to
R501 in Mrd interacts through its guanidino group with both oxygen atoms of a carboxyl
group of succinate, positioning it parallel to the isoalloxazine ring [16]. Mrd does not have two
other conserved residues that interact with succinate or fumarate. It has a tryptophan instead
of histidine at position 311 and valine instead of serine or threonine at position 324. Since
these amino acids are also involved in substrate binding, it is possible that their absence is due
to substrate specificity of Mrd of G. sulfurreducens AM-1.

Biogenesis of chromoproteins of the methacrylate redox system probably occurs via differ-
ent mechanisms. The immature Mrd protein has a longer and less hydrophobic Tat-type signal
peptide sequence (Table 1, Fig 3), characteristic for Bacteria, Archaea and chloroplast proteins.

Fig 5. An unrooted phylogeny reconstruction of 16s RNA from the strains coding for mrd and mcc homologs or from their closest relatives. The branch on which the horizontal gene transfer of the operon carrying the mrd and mcc genes has occurred is indicated by a red mark.

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Such proteins are transported through the membrane after folding [45]. A Sec-type signal peptide sequence was found in the immature Mcc protein (Table 2, Fig 4). Such proteins are translocated across the membrane before the acquisition of tertiary structure [45,46], with heme attachment occurring in the periplasm [37,46]. Thus, both the Tat- and Sec-type secretory mechanisms are likely to be required for maturation of the methacrylate redox system proteins.

Genes of the methacrylate reductase system components of G. sulfurreducens AM-1 are organized similarly to genes for their closest homologs in four representatives of δ-Proteobacteria and one representative of Deferribacteres (see RESULTS). Thus, it is possible that these organisms may also either be able to grow using methacrylate as a terminal electron acceptor or at least show some methacrylate-reducing activity.

The methacrylate reductase system is representative of a comprehensive family of flavocytochromes c and flavoproteins with reducing properties. These reducing complexes probably use a natural substrate, for example, acrylate produced by marine bacteria [26,47]. The rates of reduction of acrylate and methacrylate by the methacrylate reductase system are comparable [18] supporting the hypothesis of the use of some natural substrate by these proteins. Methacrylate reduction may be an additional characteristic of this reductase system.

Components of the methacrylate reductase system from G. sulfurreducens AM-1 and lyase of dimethylsulphoniopropionate (DMSP) of DddY-type from marine microorganisms have some similar features: 1) a distribution in certain groups of proteobacteria, 2) gene organization with cytochrome c genes adjacent to the enzyme genes (reductase or lyase) and 3) presence of cleavable signal peptide in the immature enzymes. The enzyme DddY catalyzes the cleavage of DMSP to the volatile compound dimethyl sulphide (DMS) and the toxic acrylate [47]. We suggest that the reductase evolved to transform the toxic acrylate, formed by lyases, to a less toxic compound. These cytochromes c, whose genes are located near the reductase or lyase genes, may be homologous.

The methacrylate reductase system evolved from a cytochrome c and a flavoprotein. These proteins were recently acquired by horizontal gene transfer by G. sulfurreducens AM-1 either before or after the evolution of the substrate specificity. Furthermore, these proteins likely constitute an adaptive mechanism to allow growth in sludge microbial communities, in particular, in wastewater of plastic manufacture factories.

Experimental Procedures

The object of investigation was anaerobic bacterium G. sulfurreducens AM-1 from the culture collection of Laboratory of microorganisms adaptation at the Institute of Biochemistry and Physiology of Microorganisms (Pushchino, Russia).

The subject of investigation was the operon containing genes mrd and mcc of the methacrylate redox system of G. sulfurreducens AM-1.

Genome sequencing

The draft genome sequences were obtained by pair-end library and mate pair library sequences by Illumina HiSeq 2000. The resulting contigs were submitted to GenBank under the accession numbers of CP010430.

Genome assembly

The genome was assembled de novo using SOAPdenovo [48], Velvet [49] and SPAdes Genome Assembler [50]. The quality of assembly was estimated by running QUAST [51] and by aligning of the contigs to the full genomes of Geobacter sulfurreducens available in GenBank.
Geobacter sulfurreducens KN400 and Geobacter sulfurreducens PCA. The alignments were done with Mauve [52].

The contigs obtained by SPAdes turned out to be the best. Nevertheless, SPAdes failed to assemble the genome into one sequence. We used SSPACE [53] for scaffolding. This allowed us to obtain the genome as just one contig. After this we applied GapFiller [54] for closing gaps.

Sequence analysis

Detection of the mrd and mcc genes of the bacterium G. sulfurreducens AM-1 and comparative amino acid analysis were performed with the BLAST program [55] from the National Center of Biotechnology Information server, National Library of Medicine, USA (NCBI; http://www.ncbi.nlm.nih.gov).

Analysis of nucleotide sequences of the studied operon was carried out using the Vector NTI program [56]. The presence and types of promoters and terminators were detected with a series of programs, available on the site http://linux1.softberry.com.

The sequencing of the mrd start and mrd flanking regions was performed by the Sanger method with oligonucleotide primers FA2 (5‘−ACGCTTCTCAACCAGACGG) and RA2 (5’−CATCGGTCCAAGGTTATATTCAC). Amplification for the nucleotide sequencing was performed by the PCR method using oligonucleotide primers—FG1 (5’−CAGAACAGGCCACGCTTTGG) and RG1 (5’−GTCGGGAACCAACTGCTGCCC).

All amino acid sequences of proteins and nucleotide sequences of genes are available in the Databases GenBank, Gene, Genome, Nucleotide, Protein from the server of the NCBI.

Determination of the cleavable signal peptides was conducted with the programs PRED-TAT [45] and SignalP [57], available on servers of the Department of Computer Science and Bio-medical Informatics, University of Central Greece, Lamia, Greece (http://www.compgen.org) and the Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark (http://www.cbs.dtu.dk).

Program GENERUNR (http://www.generunner.net) was used for the detection of conserved amino acid sequences and calculation of molecular weight. The number of hemes in homologous proteins was predicted as number of heme-binding sites CXXCH (where C is cysteine, H is histidine, X is any amino acid) [37].

Multiple protein sequence alignment of methacrylate redox system components and their homologs was performed with MUSCLE [58]. Phylogenies were reconstructed using the MrBayes v3.2 program [59], with mcmc = 3000000 and burnin = 2500 for sump and sumt.

Acknowledgments

Our author Dr., Prof. Akimenko Vasilii K. (1942–2013) passed away during work on the article. Prof. Akimenko was a leading biochemist in IBPM RAS and active researcher until last days. A number of his work remains unfinished. We mourn premature care of Prof. Akimenko Vasilii.

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Author Contributions

Conceived and designed the experiments: OVA MVM GVM MVZ ASG VKA FAK. Performed the experiments: OVA MVM GVM MVZ FAK. Analyzed the data: OVA MVM GVM MVZ ASG FAK. Contributed reagents/materials/analysis tools: OVA MVM GVM MVZ FAK. Wrote the paper: OVA MVM ASG FAK.
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