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Evolution of the syntrophic interaction between *Desulfovibrio vulgaris* and *Methanosarcina barkeri*: Involvement of an ancient horizontal gene transfer

Johannes C. Scholten [a,*], David E. Culley [a], Fred J. Brockman [a], Gang Wu [b], Weiwen Zhang [a,*]

[a] *Microbiology Department, Pacific Northwest National Laboratory, P.O. Box 999, Mail Stop P7-50, Richland, WA 99352, USA*
[b] *Department of Biological Sciences, University of Maryland at Baltimore County, Baltimore, MD 21250, USA*

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Abstract

The sulfate reducing bacteria *Desulfovibrio vulgaris* and the methanogenic archaea *Methanosarcina barkeri* can grow syntrophically on lactate. In this study, a set of three closely located genes, DVU2103, DVU2104, and DVU2108 of *D. vulgaris*, was found to be up-regulated 2- to 4-fold following the lifestyle shift from syntroph to sulfate reducer; moreover, none of the genes in this gene set were differentially regulated when comparing gene expression from various *D. vulgaris* pure culture experiments. Although exact function of this gene set is unknown, the results suggest that it may play roles related to the lifestyle change of *D. vulgaris* from syntroph to sulfate reducer. This hypothesis is further supported by phylogenomic analyses showing that homologies of this gene set were only narrowly present in several groups of bacteria, most of which are restricted to a syntrophic lifestyle, such as *Pelobacter carbinolicus*, *Syntrophobacter fumaroxidans*, *Syntrophomonas wolfei*, and *Syntrophus aciditrophicus*. Phylogenetic analysis showed that all three individual genes in the gene set tended to be clustered with their homologies from archaeal genera, and they were rooted on archaeal species in the phylogenetic trees, suggesting that they were horizontally transferred from archaeal methanogens. In addition, no significant bias in codon and amino acid usages was detected between these genes and the rest of the *D. vulgaris* genome, suggesting the gene transfer may have occurred early in the evolutionary history so that sufficient time has elapsed to allow an adaptation to the codon and amino acid usages of *D. vulgaris*. This report provides novel insights into the origin and evolution of bacterial genes linked to the lifestyle change of *D. vulgaris* from a syntrophic to a sulfate-reducing lifestyle.

Keywords: Syntrophy; Gene transfer; *Desulfovibrio vulgaris*

Unlike eukaryotes that evolve principally through the modification of existing genetic information, bacteria can obtain a significant proportion of their genetic diversity through the acquisition of sequences from distantly related organisms. Horizontal gene transfer produces extremely dynamic genomes in which substantial amounts of DNA are introduced into and deleted from the chromosome. These lateral transfers have effectively changed the ecological and pathogenic character of bacterial species [1]. Recent studies indicated that the scale of horizontal gene transfer may have previously been underestimated and that horizontal gene transfer may be a major force in the evolution of prokaryotic genomes [2,3].

Microorganisms in their ecological niches in nature rarely exist alone. One of the most remarkable types of microbial interactions is termed "syntrophy", which translates as "feeding together" [4]. For example, in the absence of sulfate, neither *Desulfovibrio vulgaris* nor the methanogen *Methanosarcina barkeri* can individually grow on lactate as the sole carbon source. However, by forming a

* Corresponding authors. Fax: +1 509 372 1632.
E-mail addresses: Johannes.Scholten@pnl.gov (J.C. Scholten), Weiwen.Zhang@pnl.gov (W. Zhang).

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syntrophic relationship, these two organisms can work cooperatively to utilize this compound even in the absence of sulfate. This syntrophic interaction involves lactate oxidation by *D. vulgaris* to produce acetate, CO_2, and H_2 as products, which the methanogen, *M. barkeri*, can then further convert to CH_4. The removal of H_2 by *M. barkeri* is particularly important in this interaction, as the concentration of H_2 in the vicinity of *D. vulgaris* must be kept at extremely low levels to provide a thermodynamically favorable condition for the continued oxidation of lactate [5,6]. Syntrophy has been considered as the heart of how methanogenic and other anaerobic microbial communities function [7].

We have been studying the syntrophic interaction between *D. vulgaris* and *M. barkeri* for the past several years [8–10]. Such efforts have led to the discovery of several dozen *D. vulgaris* genes that are potentially involved in syntrophic growth with *M. barkeri* (Scholten et al., unpublished data). In this study, we report the finding from microarray and real-time PCR analyses that a functionally unknown gene set in the *D. vulgaris* genome, which consists of three closely located genes on the chromosome, may be involved in the lifestyle change of *D. vulgaris*. Two of these genes, DVU2103 and DVU2104, encode iron-sulfur cluster-binding/ATPase domain proteins and the third, DVU2108 which was located upstream of the DVU2103 and DVU2104, encodes an MTH1175-like domain family protein. By integrating phylogenomics, phylogenetic, codon and amino acid usage analyses, we discovered that this gene set in *D. vulgaris* seems to be involved in during its lifestyle change from syntroph to sulfate reducer, and their presence in *D. vulgaris* is a result of an ancient horizontal gene transfer from an archaeal methanogen. This report provides novel insights into the origin and evolution of genes involved in *D. vulgaris* lifestyle change, and might help to better understand syntrophic relationships at the molecular genetic level.

Experimental procedures

Experimental design. Desulfovibrio vulgaris subsp. *vulgaris* (DSM 664) and *M. barkeri* Fusaro (DSM 804) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and grown in bicarbonate-buffered, sulfide-reduced mineral medium as described previously [11]. Pure cultures of *D. vulgaris* and *M. barkeri* were maintained on lactate (40 mM) with sulfate (20 mM) and hydrogen, respectively. Continuous cultivation experiments with co-cultures of *D. vulgaris* and *M. barkeri* were performed in duplicate in 7.5-l Bioflow chemostat fermentors (New Brunswick) as described previously [11] with a working volume of 4000 ml at 35 °C. The dilution rate of the chemostats was set at 0.4 d^{-1}. In order to study the lifestyle change of *D. vulgaris* from syntroph to sulfate reducer, co-cultures were initially grown under lactate limitation and absence of sulfate i.e., syntrophic conditions. These syntrophic co-cultures were then perturbed by adding an excess of sulfate (15 mM final) to the chemostats and medium carboys and were further grown under lactate limitation/sulfate excess condition i.e., sulfidogenic conditions. Cells from the syntrophic phase ($T = 34$ days), perturbation phase (2 h after perturbation, $T = 48$ days and 2 h) and two time-points from the sulfidogenic phase ($T = 52$ and 83 days, respectively) were collected for microarray analysis. Steady-state conditions for both the syntrophic and sulfidogenic phase were maintained for at least six volume changes. Substrate and product conditions were monitored, and total cell mass and species composition were checked under all steady-state conditions [11].

Microarray analysis. Oligonucleotide microarrays were designed by NimbleGen Systems, Inc. (Madison, WI) [12,13]. The raw intensity data were normalized using tools available through the Bioconductor project (http://www.bioconductor.org). For each experimental condition, mRNA abundances were determined from the average of four measurements for each gene: two replicates (each containing a pool of three biological replicates) that were each hybridized to duplicate microarrays [8].

Real-time PCR analysis. Verification of the microarray results for the DVU2108 gene was performed using TaqMan quantitative real-time PCR (qPCR). Briefly, samples of RNA from each time-point used in the microarray analysis were converted to cDNA using random primers and the StrataScript qPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA) according to the manufacturers' instructions. qPCR was then performed using the amplification primers DVU2108-F (5′-CACAGGGGGCGG GCATTCAG-3′), and DVU2108-R (5′-TGTCGGCAGGGCGGAGTT TG-3′), and the probe oligo DVU2108-Probe (5′-6-Fam-CGCCGTGT ACCTGTGCGACCTCGCC–TAMRA-3′). The reactions were carried out in an ABI 7700 Sequence Detector (Perkin-Elmer, Foster City, CA) using the Brilliant qPCR Master Mix Kit from Stratagene according to the manufacturers' instructions. The reaction conditions used were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 25 s and 60 °C for 1.5 min. The resulting C_t data was normalized relative to 16S rRNA levels using the $\Delta\Delta C_t$ method as described in the Applied Biosystems User Bulletin #3 (Perkin-Elmer, Foster City, CA).

Phylogenomics and phylogenetic analysis. Deduced protein sequences of DVU2103, DVU2104, and DVU2108 were used to search NCBI protein database at http://www.ncbi.nlm.nih.gov/BLAST/ and the Comprehensive Microbial Resource of The Institute for Genomic Research at http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi using *Blastp* and PSI-*Blast*. The homologies were subjected to further confirmation by domain identification. Domain identification was performed using the molecular architecture research tools provided by SMART (http://smart.embl-heidelberg.de/) [14] or Clusters of Orthologous Groups of proteins (COGs) by NCBI (http://www.ncbi.nlm.nih.gov/COG/) with *E*-value <0.01 [15]. The STRING database, which includes known and predicted protein–protein interactions (http://string.embl.de/) [16], was used to check for potential protein interactions. Sequence alignments were performed using default parameters of the ClustalW program (Version 1.82) [17] available from EMBL-European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/) (Protein Gap Open Penalty = 10.0, Protein Gap Extension Penalty = 0.2, Protein matrix = Gonnet). Multiple alignments were modified after visual examination, and a minimum number of gaps were inserted manually in order to produce what looked like a reasonable alignment. Alignments were subjected to two phylogenetic tree-building methods: Neighbor Joining [18] and Maximum Parsimony implemented in the program PAUP, Version 4.0 (Phylogenetic Analysis Using Parsimony, beta version, Sinauer Associates, Sunderland, MA). In addition, 1000 replications of bootstrap sampling were also performed as described previously [19].

Codon and amino acid usage analyses. Correspondence analyses of codon and amino acid usage were performed for both genomes as described previously [20].

Results

A set of three closely located D. vulgaris genes differentially regulated during the lifestyle shift from syntroph to sulfate reducer

The syntrophic co-culture of *D. vulgaris* and *M. barkeri* was perturbed by adding sulfate to induce a change in *D. vulgaris* lifestyle from syntroph to sulfate reducer. The lifestyle change in *D. vulgaris* was confirmed by analyzing

Table 1
Gene expression change (ratio) during lifestyle shift from syntrophy to sulfate reducer

Gene ID	Sulfate reducer vs. syntrophy lifestyles[a]			Description
	48 d	52 d	83 d	
DVU2103	3.35	3.04	2.02	Iron-sulfur cluster-binding/ATPase domain protein
DVU2104	2.44	2.35	1.5	Iron-sulfur cluster-binding/ATPase domain protein
DVU2108	3.67 (3.14)	4.06	2.15 (2.16)	MTH1175-like domain family protein

[a] Ratio calculated from microarray data is indicated for all genes. The *p*-values of ratio are all less than 0.01. Ratio from RT-PCR for DVU2108 is indicated inside the parentheses.

the chemostat performance. A set of three closely located and functionally unknown genes was found with the increased transcript levels associated with *D. vulgaris* during its lifestyle change from syntroph to sulfate reducer (Table 1). Two of these genes in this gene set, DVU2103 and DVU2104, are located in one operon with open reading frames (ORF) overlapping stop and start codons [13]. Both ORFs encode iron-sulfur cluster-binding/ATPase domain proteins of unknown function and were expressed at 2- to 3-fold higher levels in sulfidogenic condition relative to the syntrophic condition. The third gene in the gene set, DVU2108, is located three ORFs upstream of the DVU2103–DVU2014. However, sequence analysis suggests that DVU2108 is not located in the same operon with DVU2103–DVU2104 in the *D. vulgaris* genome. The gene encodes a MTH1175-like domain family protein of unknown function [13], and was expressed at 3- to 4-fold higher levels in sulfidogenic condition relative to the syntrophic condition. Up-regulation of DVU2108 in sulfidogenic condition was also confirmed by real-time PCR (Table 1). This result, along with our previous studies showing that none of the individual genes in this gene set were differentially regulated when *D. vulgaris* was grown alone in various pure culture conditions [8,9], supports the hypothesis that these three genes are important in *D. vulgaris* lifestyle change from syntroph to sulfate reducer.

Domain analyses

Domain analysis showed that although DVU2103 and DVU2104 shared only 41% similarity (29% identity), both genes are of equal-length and contain an identical full-length CobQ/CobB/MinD/ParA nucleotide binding domain with high confidence (*E*-value $< e$-17) by the SMART domain analyses or full-length COG1149 domain (MinD superfamily P-loop ATPase containing an inserted ferredoxin domain) by NCBI COG analysis. This domain has been found to be present in various cobyrinic acid a, c-diamide synthases, such as CbiA and CbiP from *Salmonella typhimurium* [21], and in CobQ from *Rhodobacter capsulatus* [22]. This family also contains dethiobiotin synthetases as well as the plasmid partitioning proteins of the MinD/ParA family [23].

Domain analysis showed that DVU2108 contains a full-length MTH1175 domain that is part of a family of conserved hypothetical proteins (COG1433) with unknown functions consisting of multiple paralogs from all complete archaeal genomes and the archaeal gene-rich bacterium *Thermotoga maritima*. Sequence similarity indicates this protein family may be related to the nitrogen fixation proteins NifB and NifX [24].

Phylogenomic analyses

We applied a phylogenomic approach to investigate the distribution patterns of all three genes across microbial genomes [25]. The initial analysis was performed using STRING, a database of known and predicted protein–protein interactions from 179 microbial species [16]. The results from this analysis showed obvious clustering of these genes in most archaeal genomes and several bacterial genomes (data not shown). We then performed a more complete search of the NCBI sequence database (523 bacterial/28 archaeal/106 eukaryotic genomes as of April 26, 2006) and TIGR CMR database (279 bacterial/23 archaeal genomes as of April 26, 2006) using *Blastp* and PSI-*Blast*. Several observations made from examination of similarity searching were: (i) because the CobQ/CobB/ParA nucleotide binding domain and COG1149 domain (MinD superfamily P-loop ATPase containing an inserted ferredoxin domain) in either DVU2103 or DVU2104 are present in functionally diverse genes, *Blast* search using either of these two sequences pull out a large number of homologies (more than 100 equal-length homologies across multiple microorganism species) even using a stricter *E*-value criterion of 0.0001. In contrast, equal-length homologies of DVU2108 which contain only MTH1175 domain were much narrowly distributed, and were found only in 30 species even using *E*-value cutoff of 0.5. Among them 28 species have full genome sequences available, while two species *D. gigas* and *Chlorobium phaeobacteroides* with only partial genome sequences; (ii) except for *D. gigas* and *Ch. phaeobacteroides* whose full genome information is not available for checking the existence of DVU2103–2104 homologies, all rest 28 species with DVU2108 homologies contained DVU2103 and DVU2104 homologies as well (Fig. 1). Among them, *Desulfotomaculum reducens* has two copies of the gene set, both with the same genetic organization possibly as a result of gene duplication (see phylogenetic analysis below); (iii) in almost all 28 cases, homologies of these three genes were closely associated on the chromosomes. Homologies of

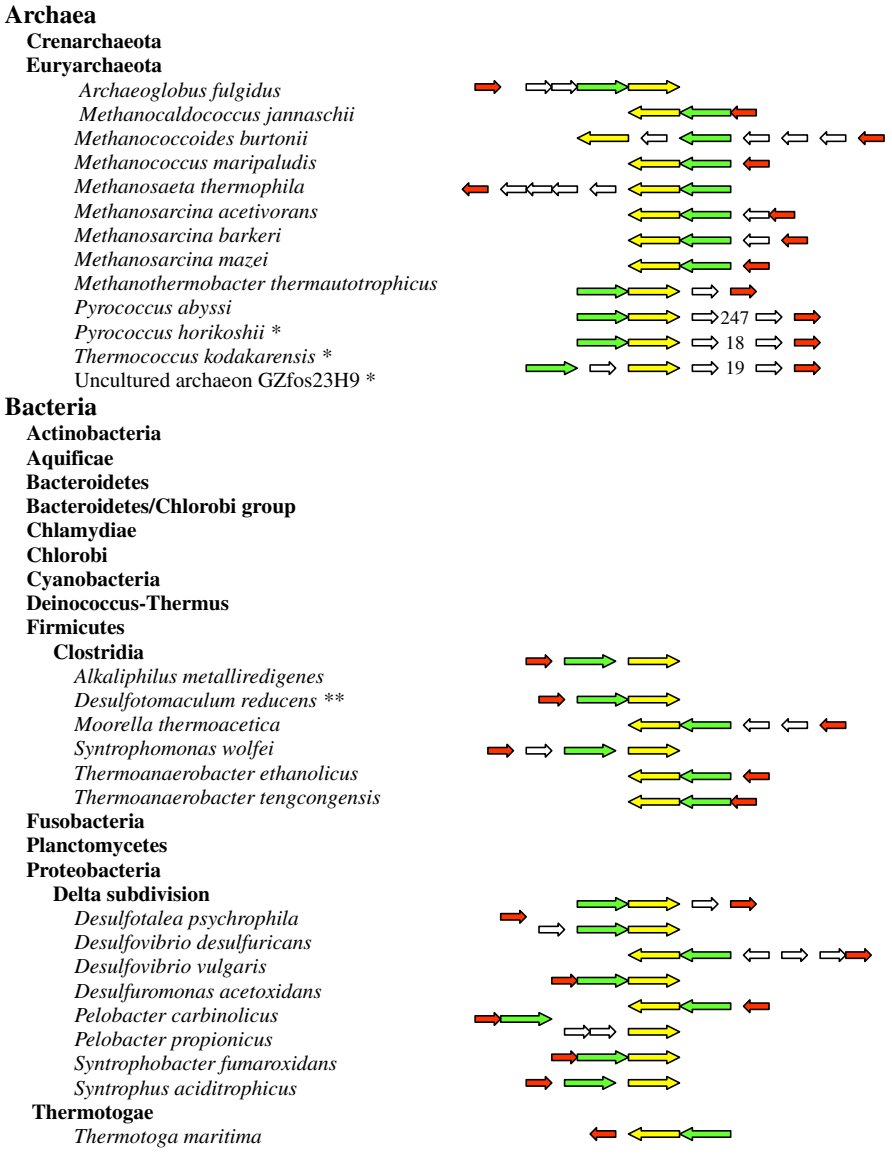

Fig. 1. Phylogenomic analysis of the DVU2103, DVU2104, and DVU2108 genes and their homologies in 28 microbial species. Presence of the genes was indicated if the score of blasting search (*E*-value) is less than 0.5. Homologies of DVU2103, DVU2104, and DVU2108 are indicated by yellow, green, and red, respectively. The sizes of the genes are not drawn to scale. *The number of ORFs in between is indicated. **_Desulfotomaculum reducens_ has two copies of the gene set, both with the same genetic organization as showed in the figure. The direct boarding of ORFs in the figures indicate overlapping of start and stop codons, while space between ORFs indicates intergenic region larger than 1 base pair.

DVU2103 and DVU2104 were directly adjacent to each other in 25 genomes and; among these, 20 genomes possessed overlapping start and stop codons for these genes. The only exceptions to this pairing of the DVU2103 and DVU2104 homologies were found in *Methanococcoides burtonii*, *Pelobacter propionicus* and the uncultured archaeon GZfos23H9 where these genes were separated by a single intervening gene (Fig. 1). Homologies of DVU2108 were normally located within the neighborhood of 2–3 ORFs of those of the DVU2103–DVU2104 homologies, DVU2108 was located in the same operon as DVU2103 and DVU2104 in three archaea, *Methanocaldococcus jannaschii*, *Methanococcus maripaludis*, *Methanothermobacter thermautotrophicus* and eight bacteria, *Alkaliphilus metalliredigenes*, *Desulfuromonas acetoxidans*, *Pelobacter carbinolicus*, *Syntrophobacter fumaroxidans*, *Syntrophus aciditrophicus*, *Thermoanaerobacter ethanolicus*, *T. tengcongensis*, and *Thermotoga maritima* (Fig. 1). The consistent clustering pattern of these three genes across taxonomically diverse microorganisms suggested that they may have shared a similar evolutionary course and may be involved in common cellular functions; and (iv) Phylogenetically, homologies of this gene set were present in almost all members of *Euryarchaeota* family of archaea, while it was only found in the bacterial genera Clostridia, δ-Proteobacteria, and Thermotogae (Fig. 1). Interestingly, almost all of the bacterial species with homologies to this gene set are known to be partners in a syntrophic interac-

Table 2
Bacteria possibly involved in syntrophic lifestyle

Microorganisms	Syntroph	Closest syntrophic relative	Substrate	Syntrophic partner	Reference
Firmicutes					
Clostridia					
Desulfotomaculum reducens		*Desulfotomaculum thermobenzoicum*	Propionate	*Methanothermobacter thermoautotrophicus*	[31]
Moorella thermoacetica		*Moorella mulderi*	Methanol	*Methanothermobacter thermoautotrophicus*	[32]
Syntrophomonas wolfei	Yes		Butyrate	*Methanospirillum hungatei* [a]	[5]
Proteobacteria					
δ Subdivision					
Desulfotalea psychrophila					
Desulfovibrio desulfuricans		*Desulfovibrio vulgaris*	Lactate	*Methanosarcina barkeri*	[6]
Desulfovibrio vulgaris	Yes		Lactate	*Methanosarcina barkeri*	[6]
Pelobacter carbinolicus	Yes		Ethanol	*Methanospirillum hungatei*[a]	[33]
Pelobacter propionicus		*Pelobacter carbinolicus*	Ethanol	*Methanospirillum hungatei*	[34]
Syntrophobacter fumaroxidans	Yes		Propionate	*Methanospirillum hungatei*	[35]
Syntrophus aciditrophicus	Yes		Benzoate	*Methanospirillum hungatei*[a]	[36]
Thermotogae					
Thermotoga maritima		*Thermotoga lettingae*	Acetate	*Methanothermobacter thermoautotrophicus*[a]	[37]

[a] Other syntrophic partners have also been reported for this organism than the one mentioned here in the table.

tion with a methanogen as shown in Table 2. All above observations, along with our microarray results from the co-cultures of *D. vulgaris* and *M. barkeri* showing that all three genes were all up-regulated during *D. vulgaris* lifestyle change from syntroph to sulfate reducer but that none of these genes were differentially regulated when *D. vulgaris* was grown alone in various pure culture conditions, led to our suggestion that it is the gene set, rather than individual gene, could be an important feature related to syntrophic relationships.

Phylogenetic analyses

The narrow distribution pattern of this gene set did not follow the evolutionary and taxonomic relationships of these microorganisms, strongly suggesting that horizontal gene transfer may have been involved in its origin and evolution. To test this possibility, phylogenetic trees were constructed using complete coding sequences of DVU2103, DVU2104, and DVU2108 and their homologies from the 28 species which contained the gene set. The trees were constructed by both Neighbor Jointing (NJ) and Maximum Parsimony (MP) methods and evaluated by 1000 bootstrap replicates (NJ trees shown in Supplementary Fig. 1). Despite the resolution of the phylogenetic analyses being restricted by the low ratio between the number of aligned residues and the number of operational taxonomic units (OTUs) in the datasets, the key clusters in phylogenetic trees of DVU2108 and DVU2103–DVU2104 were supported by greater than 75% of 1000 random bootstrap replicates (Supplementary Fig. 1).

The conventional view of the universal evolutionary tree is that bacteria and archaea are two separated branches from a common ancestor [26]. However, the phylogenies we derived for DVU2103–DVU2104 and DVU2108 deviate from this canonical view since they clearly cluster with their archaeal homologies in both trees. Moreover, based on their phylogenies, these sequences appear to be rooted in archaea (Supplementary Fig. 1). This evidence supports the hypothesis that the gene set of DVU2103, DVU2104, and DVU2108 may have originated in archaea after archaea were separated from their last common ancestor with bacteria and later radiated into bacteria through horizontal gene transfer. In addition, genes from each group of microorganisms, archaea, δ-Proteobacteria and Clostridia tend to be clustered together in the trees; moreover, each sub-clade in the DVU2108 tree are congruent with that in the DVU2103 or DVU2104 trees, suggesting these genes belonged to a functionally related gene set.

Codon and amino acid usage analyses

Analyses of codon usage patterns or amino acid usage pattern have been used as methods to identify horizontal gene transfers [27]. Recent studies have shown that amino acid usage may also be different between genes acquired by horizontal gene transfer and its host genomes [28]. However, when correspondence analyses were performed to compare the codon usage and amino acid usage patterns between DVU2103–DVU2104–DVU2108 and the rest of the *D. vulgaris* genome, no significant bias was observed for either their codon or amino acid usage (Supplementary Fig. 2). In addition, G + C content analysis showed no difference between the genes and the remainder of the *D. vulgaris* genome. These results support the suggestion that the transfer might have occurred in the early

evolutionary history of *D. vulgaris* and that an adaptation to the codon and amino acid usage patterns of its host has been completed.

Discussion

Although DVU2108 is not located in the same operon with DVU2103–DVU2104 in *D. vulgaris*, the homologies of this gene set are found present as one operon in three archaeal and eight other bacterial species, suggesting it is possible that homologies of this gene set were originally located in one operon in ancient methanogens and transferred as one operon into bacteria. The genes were then subjected to minor modifications to their relative position in the host chromosome during microbial speciation. It has been shown that xenologous gene displacement, that is, displacement of an ancestral gene by a horizontally transferred ortholog from a taxonomically distant organism without change of the local gene organization, or independent assembly (part from horizontally acquired genes) are commonly involved in horizontally acquired operons [29], however, this does not appear to be the case for the set of three lifestyle change related genes identified in this study since they all seem to have shared a common evolutionary history. Their consistent clustering pattern across taxonomically remotely related species and the congruent patterns in their phylogenetic trees suggest that all three genes were functionally related and their horizontal transfers occurred as a single event for all three genes. Our data supports the "selfish operon hypothesis" that states that gene clusters are created and maintained by selection for transferability [30]. This is consistent with our hypothesis that this set of three genes is required to perform a function related to a lifestyle change from syntroph to sulfate reducer, and that only the acquisition of all of these genes at one time could give the selective advantage required to maintain them.

The scattered presence of the archaeal-like genes among the distantly related genera Clostridia, δ-Proteobacteria, and Thermotogae suggested three alternative interpretations of horizontal gene transfer: (i) multiple horizontal gene transfers might have occurred for each genus of bacteria separately; (ii) one genus of bacteria originally acquired these genes, which was thereafter independently transferred from that bacteria to other genera of bacteria; and (iii) only one single horizontal gene transfer was involved between archaea and a common ancestor of the genera of Clostridia, δ-Proteobacteria, and Thermotogae at very early stage of their evolutionary courses, followed by loss of this gene set in many bacterial lineages during their subsequent evolution. It is still unclear which of these routes are responsible for origin of the gene set in bacteria. In addition, phylogenetic analysis suggested the DVU2103–DVU2104 homologies not located within the gene set were also clustered with archaeal species, and quite possibly as results of horizontal gene transfer (data not shown); however, it may be worth further investigation whether they were results of selective gene duplication of the DVU2103–DVU2104–DVU2108 gene set or of gene loss (DVU2108 homology) from this gene set.

By transferring and maintaining certain genes involved in different lifestyles from archaeal to bacterial partners, one would expect that there must be some metabolic and regulatory advantages for them to improve the ability to form a partnership in their environmental niche. Without further study, it is difficult to speculate what advantage this gene transfer may have conferred or what the exact functions of these genes might be. Based on the analysis from the STRING database, which predicts various protein–protein interactions [16], homologies of this gene set seem to be involved in aspects of major metabolic pathways in these organisms e.g., methanogenesis for most of the methanogenic archaea. For example, homologies of this gene set in *Methanosarcina acetivorans* were predicted to be directly related to the function of formylmethanofuran dehydrogenases (Supplementary Fig. 3). So far, the only known syntrophic bacterium in the STRING database is *D. vulgaris*, where this gene set is predicted to be involved in some aspect of chemotaxis (Supplementary Fig. 4). However these deductions are still very speculative and more data will be needed to verify this hypothesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.164.

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