Research Paper

Evidence of methanesulfonate utilizers in the Sargasso Sea metagenome

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Methanesulfonate (MSA) is one of the products of the photo-oxidation of dimethylsulfide in the atmosphere. The genes responsible for the import of MSA into the cell (*msm*EFGH) and for its oxidation to formaldehyde (*msm*ABCD) have been previously sequenced from the soil bacterium *Methylosulfonomonas methylovora* str. M2 while genes for an MSA monooxygenase have been sequenced from marine bacterium *Marinosulfonomonas methylotropha* str. TR3. We performed a sequence-based screening of the Sargasso Sea metagenome for homologues of the MSA monooxygenase (MSAMO) and MSA import genes. Our search retrieved one scaffold bearing genes with high identity to the *msm*ABCD cluster plus two scaffolds bearing genes highly identical to the *msm*EFGH operon. We increased the available data by sequencing two metagenome plasmids, which revealed more *msm* genes. In these three cases synteny with the original *msm* operons was revealed. We also retrieved several singletons showing high identity to shorter segments of the *msm* clusters or individual *msm* genes. Furthermore, a characteristic 26-aa internal spacer of the MsmA Rieske-type motif was conserved. Our findings support the case for a significant role of MSA degraders in the marine sulfur cycle and seem to suggest that they may be prominent members of the methylotrophic community in surface ocean waters.

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Introduction

Methanesulfonic acid (MSA) is a compound naturally produced in the atmosphere (estimated at ca. 10^{10} kg/year) by the photo-oxidation of dimethylsulfide (DMS), which in turn is the degradation product of dimethylsulfoniopropionate (DMSP), an osmolyte utilized by marine algae and cyanobacteria [1, 2]. The absence of accumulation of MSA in the biosphere (other than in perennial ices) demonstrates that this chemically stable compound must be actively degraded in Nature [3, 4]. Several bacterial strains capable of growing using MSA as sole source of C and energy have been isolated in the past 15 years [5–9]. In all cases analyzed, these bacteria harbored a specific multimeric enzyme, MSA monooxygenase (MSAMO), which catalyzes the first oxidative step of MSA to the central methylotrophic intermediate formaldehyde. All known MSA degraders have DNA with a relatively high G + C content (57 to 67%), they are serine methylotrophs belonging to the Alphaproteobacteria, albeit to a wide range of genera (*Methylosulfonomonas*, *Marinosulfonomonas*, *Methylobacterium*, *Hyphomicrobium*, *Pedomicrobium* and *Afipia*) within the Rhizobiales or the Rhodobacterales, most have been isolated from soil but also from sea water. Genes coding for the MSAMO enzyme (*msm*ABCD encoding large and small subunits of hydroxylase, ferredoxin and reductase, respectively) have been cloned and sequenced from *Methylosulfonomonas* (str. M2) and from *Marinosulfonomonas* (str. TR3), a soil and a marine strain, respectively [6, 10]. In the other MSA-utilizers for which molecular data are available, similar *msm* genes have been

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consistently detected by hybridization or by PCR [6–8]. The MSAMO genes in *Marinosulfonomonas* str. TR3 were found to be distributed between two separate incomplete but complementary operons, the first missing the *msm*D gene and the second missing *msm*C [6]. Apart from this difference, the *msm* operons in strain TR3 showed high sequence identity and general synteny with the *msm*ABCD operon from the soil strain, *Methylosulfonomonas* str. M2 (Fig. 1).

Primers targeting conserved regions of the *msm*A gene (coding for the large subunit of the MSAMO enzyme) have been successfully used to amplify a region of this gene from both pure cultures and directly from environmental samples (soil and sea water filtrate) [6].

In all cases so far analyzed at the molecular level, MsmA exhibits a unique sequence in the region containing the Rieske-type [2Fe–2S] cluster, with an extended 26 aa spacer in the CxH-x_n-CxxH conserved motif, rather than the canonical 16–18 residues [6, 8, 10].

In *Methylosulfonomonas* str. M2 an additional cluster coding for an ABC-type MSA-import system (*msmEFGH*) was found adjacent (although divergently transcribed) to the first operon [10, 11] (Fig. 1).

In recent years a large amount of new environmental sequence has become available, most of which was provided by Venter and colleagues [12] who shot-gun cloned and sequenced ca. 1045 billion bp of DNA from surface water from the northwest Sargasso Sea, off the coast of Bermuda. The Sargasso Sea Metagenome (SSM) has already been mined for phosphate acquisition genes [13], biopolymer hydrolases [14], phytochrome photoreceptors [15], nitrite reductase and ammonia monooxygenase [16], genes for DNA modification by sulfur [17], archaeal histones [18], selenoproteins [19], arsenite oxidase [20], chitinases [21], iron-sulfur proteins [22] and RNase P RNA [23]. As for the presence of core C_1 genes, homologues of methanol dehydrogenase, methylamine dehydrogenase, and methane monooxygenase (both soluble and particulate) have been sought, but only low-similarity hits were obtained [24]. In the same work a search for 16S rRNA sequences of typical representatives of methylotrophic bacteria also yielded no high-identity matches. Nonetheless, a novel clade of methanopterin-linked C_1 transfer genes of the formaldehyde oxidation (FOX) module was found.

In order to understand the relevance of MSA degraders in the Sargasso Sea surface waters, we performed a sequence-based search of the SSM for homologues of the Msm polypeptides which allowed us to confirm their presence and to estimate their numbers.

Materials and methods

To detect *msm* genes in the SSM, we used the sequences of the 8 Msm proteins known from *Methylosulfonomonas* str. M2 (accession no. AF091716) and *Marinosulfonomonas* str. TR3 (accession nos. AF354805 and AF360864) as BLASTP queries in searches at the National Institute of Health's NCBI specific database for environmental samples. All results retrieved in this way were checked against the database of records associated with classified organisms and against the non-redundant database by BLAST to ensure that the original queries used were indeed the most similar results. *Afipia*, *Pedomicrobium*, *Methylosulfonomonas* or *Marinosulfonomonas* 16S rRNA gene sequences were also used as queries to ascertain the presence of relatives of these recognized MSA degraders in the SSM water samples. ORFs (translated) flanking the *msm* genes retrieved in the search were used as BLASTP probes against the bacterial subset of the non-redundant NCBI database to try and infer their function and possible taxonomical affiliation and thus complement the annotation of the SMM contig sequences. This was very useful for recognizing small (truncated) ORFs as positive hits since they score badly in the general BLAST searches which cause them to fail to be properly identified.

Recombinant plasmids from the Venter Institute's clones library (kind gift of C. Venter and D. Rusch) were purified using the Gene Elute™ Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) and sequenced by primer walking by StabVida, Lisbon, Portugal (Taq DyeDeoxy Terminator Cycle Sequencing and a Model 373 A gel apparatus, Applied Biosystems). Novel sequences were deposited in GenBank under accession numbers EF103447 and EF103448. Sequences were aligned and analyzed using the Vector NTI programme (Invitrogen, Carlsbad, CA, USA).

Results

Our BLASTP searches within the Sargasso Sea Metagenome database using known Msm peptide sequences as queries yielded 3 scaffolds and 19 singletons with highly significant E-values (10^{-111} to 10^{-17}). Two of the 3 scaffolds retrieved had sequencing gaps in regions where we suspected that more *msm* genes could be present. Therefore, we sequenced the two corresponding Sargasso Sea original clones (kindly donated by the Venter Institute) and produced 3758 and 1104 bp of new sequence. Indeed, more *msm* homologues were identified in these new sections of sequence (Fig. 1).

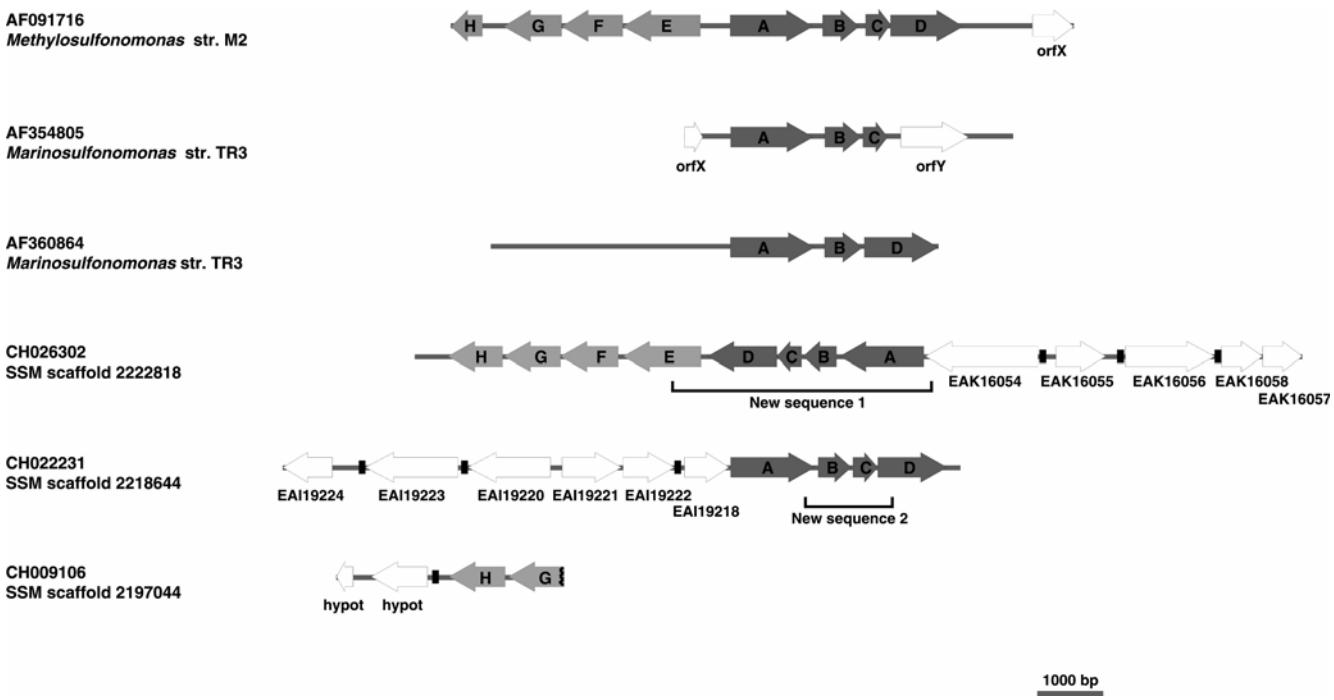

Figure 1. Graphic alignment of the msm genes from the Sargasso Sea Metagenome scaffolds and strains M2 and TR3. Dark gray arrows represent the MSA-monooxygenase cluster: *msm*A and B genes encode large and small subunits of hydroxylase; *msm*C encodes a ferredoxin; *msm*D encodes a reductase; *orf*X and *orf*Y enconde putative regulators of the *msm* operons. Light gray arrows represent the MSA-transport cluster. Black boxes represent gaps between the contigs. 1) sequence available under accession no. EF103447. 2) sequence available under accession no. EF103448.

Scaffold 2222818 (original accession no. CH026302; new record EF103447) was found to encode a complete *msm*ABCD operon with identical internal gene arrangement as in *Methylosulfonomonas* str. M2 and very similar to the order found in *Marinosulfonomonas* str. TR3, plus a full *msm*EFGH operon perfectly syntenic to the one of strain M2; one sole difference was that in this scaffold the two operons are transcribed in the same direction, whereas they are divergent in strain M2 (Fig. 1).

The second scaffold (2218644, original accession no. CH022231; new record EF103448) presents a full oxygenase operon (*msm*ABCD) with the same gene arrangement as in strain M2, but no MSA-import operon was found in the same scaffold.

A third scaffold (2197044, accession no. CH009106) was found to encode homologues of the last two of the MSA transport genes, *msm*GH.

In addition, our BLAST searches also retrieved 9 singletons bearing homologues of MSAMO components, 9 others coding for homologues of MSA-import proteins, and one coding for genes *msm*D and E; however, most of these small clones carried only incomplete segments of *msm* homologues (Table).

Seven of the singletons coded for two *msm* genes and in all cases the paired genes showed the same order and internal orientation as in the known *msm* operons.

Singleton AACY01431386 bears homologues of *msm*D (coding for the monooxygenase reductase component) and *msm*E (coding for the first component of the transport system) transcribed in the same orientation although apparently belonging to two separate operons (intergenic region of 154 bp), an arrangement identical to that found in scaffold 2222818.

Singleton AACY01326589 was found to encode a C-truncated *msm*A gene flanked by a homologue of transcriptional regulators of the LysR family. Its highest similarity score (57% similarity) was with the N-terminal region of OrfY, a putative regulator found associated to the *msm*ABD operon in *Marinosulfonomonas* strain TR3 (Fig. 1). OrfY in its N-terminal region is in turn very similar (85%) to the product of *orf*X, an incomplete gene found downstream of the oxygenase cluster in *Methylosulfonomonas* strain M2. The position of these ORFs in the genomes, consistently associated to the *msm* clusters, further strengthens the contention that *orf*X and *orf*Y are the positive regulators of the *msm* operons in the respective hosts.

Among the 8 homologues of *msm*A retrieved in our searches, all those that covered the region of the Rieske-

Table. Comparison of the *msm* (predicted) peptide sequences from the SSM.

Accession number	G+C%	msm gene present	ident (simil) to str. M2	ident (simil) to str. TR3	Rieske signature
CH026302 (sc. 2222818)	37.9	A	70 (78)	69 (77)	yes
		B	50 (63)	48 (63)	-
		C	40 (56)	46 (57)	-
		D	37 (53)	37 (53)	-
		E	48 (59)	-	-
		F	61 (75)	-	-
		G	75 (85)	-	-
		H	82 (92)	-	-
CH022231 (sc. 2218644)	39.4	A	70 (78)	69 (77)	yes
		B	50 (63)	48 (63)	-
		C	40 (56)	45 (60)	-
		D	37 (53)	37 (53)	-
AACY01389346	36.7	<A	70 (76)	67 (75)	NA
		B>	47 (64)	48 (65)	-
AACY01355705	36.2	<A	71 (76)	65 (75)	NA
		B>	49 (64)	48 (64)	-
AACY01611851	38.2	A>	72 (80)	69 (79)	NA
AACY01482438	37.6	<A>	69 (79)	70 (79)	yes
AACY01793155	37.0	A>	47 (58)	45 (56)	NA
AACY01326589	44.9	<A	71 (78)	65 (72)	NA
		orf	38 (62) *	41 (57) **	-
AACY01760721	31.4	<B	50 (63)	48 (63)	-
		C>	41 (53)	46 (57)	-
AACY01725640	28.3	D>	36 (50)	36 (52)	-
AACY01646861	55.1	<D>	37 (53)	39 (52)	-
AACY01431386	32.3	<D	39 (54)	39 (55)	-
		E>	57 (68)	-	-
AACY01273724	54.3	E>	58 (74)	-	-
AACY01709715	40.4	<E	38 (51)	-	-
AACY01689981	51.9	F	44 (62)	-	-
AACY01273725	50.2	F>	60 (75)	-	-
AACY01325347	51.3	<F	45 (62)	-	-
		G>	55 (65)	-	-
AACY01709714	44.5	G>	75 (84)	-	-
AACY01602050	52.5	<G	56 (68)	-	-
CH009106 (sc. 2197044)	52.8	<G	77 (86)	-	-
		H	80 (89)	-	-
AACY01389345	33.3	<G	74 (85)	-	-
		H>	74 (83)	-	-
AACY01308555	37.6	<H	86 (95)	-	-

Identity and similarity (in parentheses) values. Low (<40%) and high (>52%) G + C% values are shown in light or dark shade, respectively. < and > symbolize N-terminal or C-terminal truncated sequences, respectively. NA = the *msm*A gene in question did not cover the relevant Rieske region.

* = with *orf*X, only 21 aa aligned.

** = with *orf*Y.

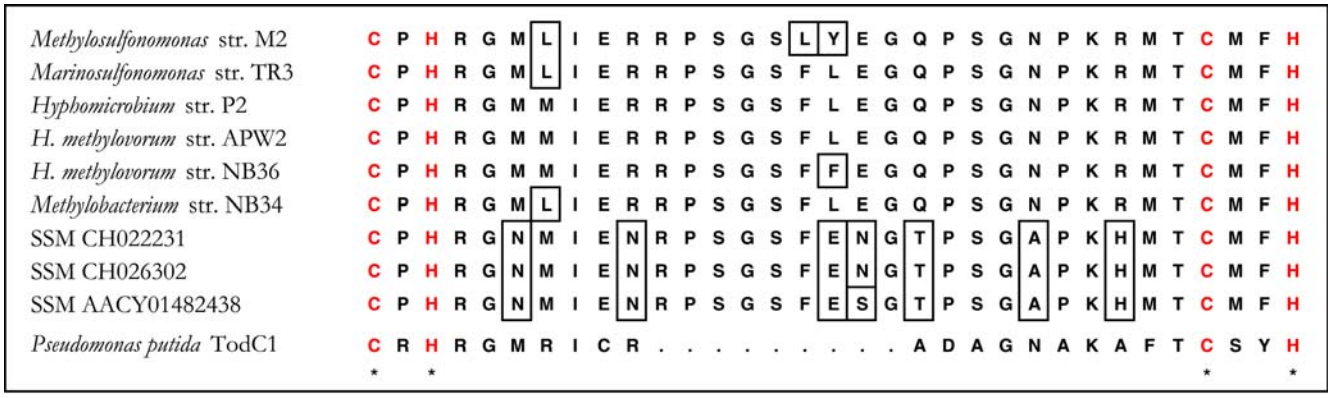

Figure 2. Polypeptide sequence alignment showing the conserved "signature" sequence in MsmA proteins and comparison to a canonical Rieske-type [2Fe–2S]-cluster motif. * indicates the conserved cysteine and histidine residues.

type [2Fe–2S] cluster motif of the protein (3 clones, two scaffolds plus one singleton) showed conservation of the peculiar extended (26 residues vs. 18–19) spacer region, a "signature" so far recognized only in hydroxylase large subunit components of MSA monooxygenases [6, 8, 10] (Fig. 2). The sequence of the spacer is surprisingly well conserved (17 residues out of 26 are invariant). Nonetheless, the 3 SSM occurrences have alternative residues that clearly set them apart from the other known cases: the M to N change (position 3), R to N (7), Q to T (17), N to A (21), R to H (24) and E at position 14 are changes found only in the three SSM records.

A major part (12 out of 22) of the *msm* gene-bearing Sargasso Sea sequences showed G + C contents below 40% (with one example as low as 28.3%), while only a minority (7) showed values above 50%, with a top value of just 55%. This matches the observation of low G + C content in the SSM, with a median of just 34% [25], while it is in stark contrast with the *msm* genes previously cloned and sequenced, which have G + C contents of 65 and 67% [6, 10, 11] and with all the known MSA utilizers, which belong to species with relatively high G + C content (59 to 65%) [6–8].

None of the *msm* sequences retrieved in this search could be explicitly assigned to a taxonomic group since none of the positive clones carried taxonomical marker genes (rRNAs, *rec*A/*rad*A, HSP70, EF-Tu or EF-G). However, the 3 scaffolds carried also non-*msm* genes that we could use (in the form of deduced polypeptide sequences) as BLASTP probes to try and infer some indication on the taxonomical position of the host organisms. The accompanying genes in scaffolds 2222818 and 2218644 showed high similarity (71–80%) to formate-tetrahydrofolate ligase and formate dehydrogenase subunits conserved across Bacteria, with the highest hits found in Alpha-, Beta- and Gamma-proteobacteria, among which some well known methylotrophic genera (*Methylobacterium*, *Paracoccus*, *Methylobacillus*, *Methylococcus*). Notably, among the best BLAST hits produced in this search were sequences belonging to bacteria of the *Roseobacter* group *Oceanicola*, *Roseovarius*, *Sulfitobacter*, and the recently characterized marine DMSP degrader *Silicibacter* [26]. However, two individual hypothetical proteins from scaffold 2218644 (EAI19223 and Orf22972309) were most similar to genes from green-sulfur bacteria (Chlorobi) and Actinobacteria. The third scaffold (2197044) bears two hypothetical genes that show similarity mainly with counterparts in the Proteobacteria (of the Alpha, Beta, Gamma and Delta sections).

Discussion

Polypeptides highly identical to those involved in MSA import and catabolism in *Methylosulfonomonas* str. M2 were found in the Sargasso Sea translated metagenome implying the presence of bacteria with the ability to use MSA as source of carbon and/or energy and/or sulfur in surface waters of the Sargasso Sea. By comparing the numbers of *msm* gene-bearing clones in the SSM (22) with the number of *rec*A genes (1029) [27], after correcting for the difference in size between the *msm* operons and the *rec*A gene, we can estimate the proportion of MSA utilizers in ca. 0.06–0.08%. The features found in these records show a high degree of conservation with genes already known: the three full *msm* operons retrieved (two *msm*ABCD and one *msm*EFGH) each was conserved in its internal structure with high values of identity/similarity to the known Msm proteins. Specifically, a characteristically long spacer in the [2Fe–2S] cluster-binding region of the hydroxylase large subunit,

distinctive of all MSAMOs known to date, was found in all applicable cases. Despite the high degree of conservation at the protein level, the environmental clones revealed a wide variability in G + C content, suggesting a great variety in the type of bacterial hosts. All MSA degraders isolated so far belong to relatively high-GC-content (57–67%) Alphaproteobacteria. The clones retrieved from the Sargasso Sea on the contrary show a majority of low GC-content sequences, with one as low as 28.3%. The observation that most of the *msm* genes found in the SSM are quite divergent (at the DNA level) from the known examples also matches the fact that no 16S rRNA gene with significant identity (above 94%) to known MSA-utilizing species (*Methylobacterium*, *Methylosulfonomonas*, *Marinosulfonomonas*, *Hyphomicrobium*, *Pedomicrobium* or *Afipia*) was identified in the SSM database ([24]; this work). Furthermore, the fact that the *msm* genes found in this metagenome were in many instances flanked by genes encoding components of enzymes involved in other C_1 pathways (formate-tetrahydrofolate ligase and formate dehydrogenase) supports the conjecture that the original hosts are methylotrophs that utilize MSA as source of energy (and possibly of carbon), and not just as a supply of sulfur. The similarity found for these accompanying genes with (among others) typical marine strains of the *Roseobacter* group is also very intriguing, since there is growing evidence that this group of bacteria is heavily involved in the cycling of organic sulfur compounds (DMSP, DMS) in sea waters [26–28].

This study demonstrates that surface waters from the Sargasso Sea contain a wide range of MSA-utilizing bacteria, which is evidence that these microorganisms are widespread and gives us clues on their diversity in the marine environment.

The variety of sequences retrieved widely expands our expectations on the diversity of this physiological group in the oceans and, more in general, in Nature and will allow the design of new PCR primers with a wider breadth of application for the detection of environmental *msm* genes.

The number of *msm* gene-bearing clones found in this screening (22) is still small to bear strong statistical significance. However, the comparison with the total lack of genes encoding the methane monooxygenases or methanol dehydrogenase or methylamine dehydrogenase from the same database [24] is striking and would seem to hint that the degradation of MSA, rather than other C1 substrates as previously reckoned, may be a major methylotrophic pathway active in ocean surface waters.

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