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In vitro reconstitution of bacterial DMSP biosynthesis

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Abstract: Dimethylsulfoniopropionate is one of the most abundant sulfur metabolites in marine environments. Biosynthesis of DMSP and degradation to dimethylsulfide are important links in the planetary sulfur cycle. In this report we provide the first complete description of a DMSP biosynthetic pathway by *in vitro* reconstitution of four enzymes from *Streptomyces mobaraensis*. Isolation of DMSP from *S. mobaraensis* cells grown at high salinity confirmed that this actinobacterium is indeed is a DMSP producing organism. The described DMSP biosynthesis follows same route as previously described in angiosperm plants. Despite this chemical congruence, limited sequence similarity between plant and bacterial enzymes suggests that that the two biosynthetic activities emerged by convergent evolution.

Dimethylsulfoniopropionate (DMSP) is one of the most abundant organosulfur compounds in marine environments with an estimated biogenic production of 10^9 tons annually.[1] DMSP is an important precursor for the production of dimethyl sulfide (DMS) which in turn is oxidized to dimethylsulfoxide (DMSO) by biotic or abiotic processes.[2] The portion of DMS that escapes to the atmosphere represents the largest natural source of airborne sulfur, second only to anthropogenic contributions.[3] DMS oxidation products aggregate to aerosols that can effect cloud formation with significant impact on the local and global climate.[4] The recent discovery that the oxidation product of DMSP – dimethylsulfoxonium propionate (DMSOP) – is also an abundant marine product added another potentially important link in the marine organosulfur cycle.[5]

As a cellular metabolite, DMSP has been implicated as a protectant against osmotic, oxidative, or thermal stress,[6] as signaling molecule,[7] or simply as a sulfur source for marine microorganisms.[8] One difficulty in studying the physiological function of DMSP is that the genetic basis for DMSP biosynthesis is poorly understood. Until recently, DMSP production was believed to be exclusive to phytoplankton, macroalgae, coral and coastal angiosperms.[1a] The recent demonstration that numerous marine α-proteobacteria also make DMSP completely changed this perspective.[9] Four different pathways for DMSP biosynthesis have been proposed based on isotope labeling and feeding experiments.[1a, 10] All four pathways start with methionine, but differ in the sequence by which S-methylation, decarboxylation, transamination and oxidation occur (Figure 1). In green algae, coral, phytoplankton and many α-proteobacteria,

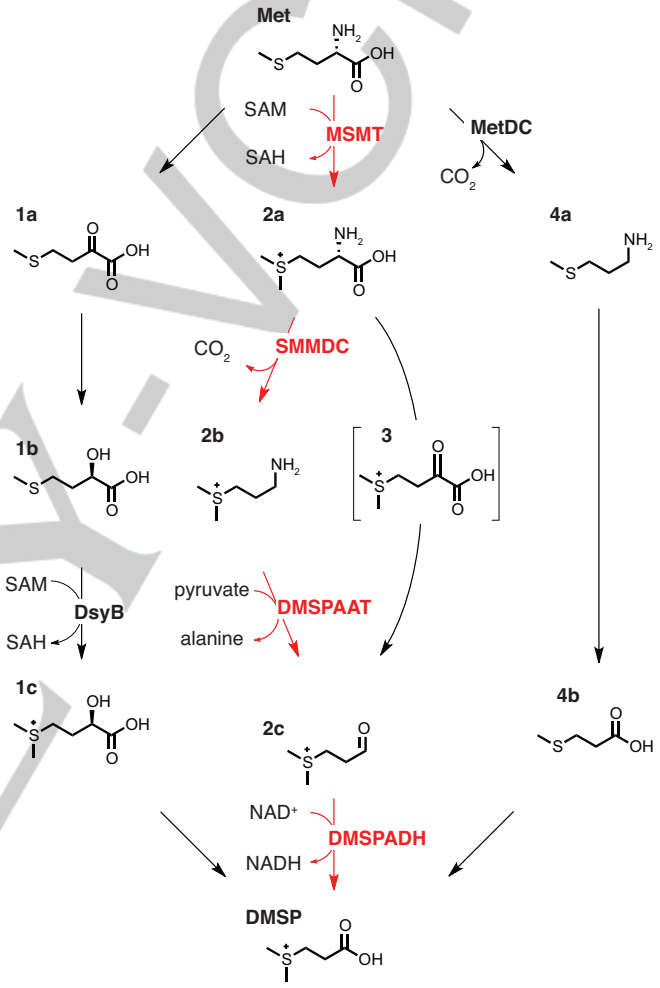

Figure 1. DMSP biosynthesis in coral, green algae, phytoplankton and many α-proteobacteria follows pathway 1 (**1a** – **1c**).[9-10] *Spartina alterniflora* (saltmarsh cordgrass),[10c, 11] and the actinobacterium *Streptomyces mobaraensis* produce DMSP via pathway 2 (**2a** – **2c**, red, this work). A third and a fourth pathway has been postulated in *Wollastonia biflora* (beach daisy, via **3**)[10d] and in Dinoflagellata (**4a** – **4b**) respectively.[10b]

the pathway starts with transamination followed by reduction, methylation, oxidation and then decarboxylation (Figure 1, route 1). Pathways described in angiosperms start with S-methylation and end with oxidation with transamination and decarboxylation occurring either as separate (route 2) or combined (route 3) steps. Detection of 3-(methylthio)propylamine (**4a**) in the dinoflagellate *Crypthecodinium cohnii* raised the possibility of a forth biosynthetic pathway (route 4).

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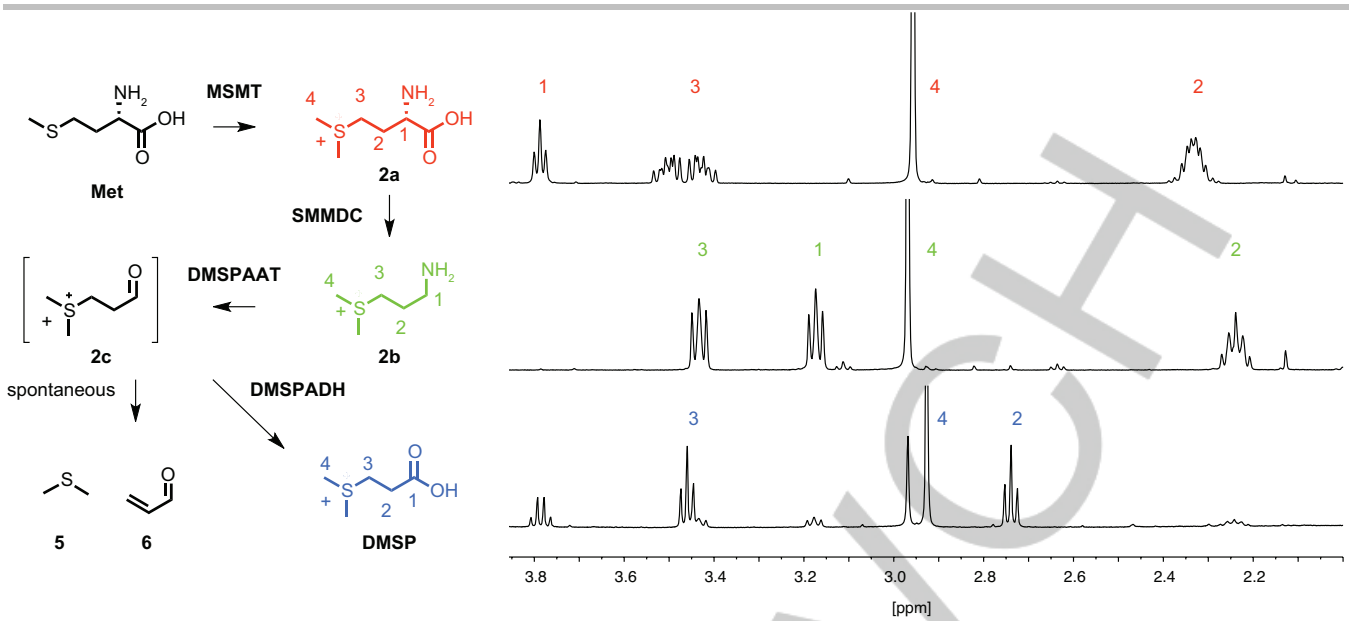

Figure 2. Product analysis of *in vitro* reconstituted DMSP biosynthesis by ^{1}H NMR spectroscopy. DMSP (blue) and the biosynthetic intermediates SMM (**2a**, red) and DMSPA (**2b**, green) were produced by the concerted activities of MSMT, SMMDC, DMSPAAT and DMSPADH. In the absence of DMSPDH the aldehyde **2c** rapidly decays to DMS (**5**) and acrolein (**6**) (Figure S8).

Despite the apparent diversity of DMSP biosynthetic pathways, information on the involved enzymes is limited.[1a] Only the methionine S-methyltransferase MSMT (route 2 and 3),[12a] and the methylthiohydroxybutyrate S-methyltransferase DsyB (route 1),[9, 14] have been identified and directly linked to DMSP production. Complete sets of DMSP biosynthetic enzymes are unknown.[15] In this report we describe the full *in vitro* reconstitution of DMSP biosynthesis from *Streptomyces mobaraensis*. This four-enzyme cascade reaction follows the same route as described for plants (route 2). However, limited sequence similarities to plant enzymes suggest that the bacterial DMSP biosynthesis emerged by convergent evolution.

Bacterial DMSP biosynthetic genes. In a BLAST search using plant MSMTs as a query we identified fourteen bacterial homologs that are encoded in conjunction with two PLP-dependent enzymes (fold-types I and III) (Table S1).[16] The relative position and direction of the three open reading frames is fully conserved (Figure S2). Most of these loci also encode a putative NAD-dependent dehydrogenase attached either before or after the conserved three-gene cluster (Figure S2). Exceptions are *Rhodovulum sp. P5* which encodes all four genes clustered on a plasmid, and S. *mobaraensis* which encodes the dehydrogenase in a different locus. Nevertheless, conserved colocalization of these genes across species from different bacterial phyla provided a first indication that the corresponding enzymes form a functional unit. To test the hypothesis that these genes enable DMSP biosynthesis, we produced the corresponding enzymes from *S. mobaraensis* as fusions with N-terminal His-tags in *Escherichia coli* and examined their catalytic activity.

Kinetic characterization of bacterial MSMTs. Because of the low sequence similarity to plant MSMTs (24 % sequence identity, seqID), we first needed to test whether the putative MSMT from *S. mobaraensis* ($MSMT_{Str}$) and from *Rhodobacter*

Table 1[a]

enzyme	$k_{cat,Met}$ [s^{-1}]	$k_{cat}/K_{M,Met}$ [$M^{-1}s^{-1}$]	$k_{cat,SAM}$ [s^{-1}]	$k_{cat}/K_{M,SAM}$ [$M^{-1}s^{-1}$]
$MSMT_{Str}$	0.09	50	0.1	230
$MSMT_{Rho}$	0.23	23	0.28	180
$MSMT_{Woll}$[b]	0.27	1900	0.27	7900

[a] The given values of the parameters represent averages from multiple independent measurements with a standard deviation less than 20% of the average value. $k_{cat,Met}$ and $k_{cat,SAM}$ were determined in the presence of 2 mM of SAM or a 20 mM of MET respectively. [b] Parameters for $MSMT_{Woll}$ were adapted from reference [12a]. The published value of V_{max} = 2.7 nkat/mg was converted to k_{cat} with a calculated mass for $MSMT_{Woll}$ of 120 kDa.

sp. JA431 ($MSMT_{Rho}$) are indeed bona fide Met-methylating enzymes. To this end, we measured the rates of S-methylmethionine (SMM, **2a**, Figure 1) production in reactions containing 1 mM methionine, 1 mM SAM and 10 uM of either enzyme. The products S-adenosylhomocysteine (SAH) and SMM were identified by chromatographic comparison with authentic samples (Figure S3). SMM was also identified by electrospray ionization high-resolution mass spectrometry (ESI HRMS, m/z calc: 164.0740; obs: 164.0740) and ^{1}H NMR (Figure 2, Table S2). To compare the bacterial enzymes with the plant MSMT from *Wollastonia biflora* (beach daisy, $MSMT_{Woll}$),[12a] we also determined the Michaelis-Menten parameters and the substrate-binding mechanism of $MSMT_{Str}$ and $MSMT_{Rho}$ (Table 1, Figure S4 and S5). This analysis revealed that the bacterial enzymes are 40-fold less efficient that the plant enzyme, mostly due to higher K_M values for both Met and SAM. However, we note that K_M values in the millimolar range are not unusual for Met-utilizing enzymes.[12b, 17] Plant and bacterial MSMTs also differ in their substrate-binding mechanism. Binding of Met and SAM to $MSMT_{Rho}$ follows a random order, whereas binding to $MSMT_{Woll}$ was

reported to follow an obligatory sequence with SAM as the leading substrate (Figure S6). These different kinetic behaviors may be an indication, that bacterial and plant enzymes were adapted to function under different conditions.

***In vitro* reconstitution of DMSP biosynthesis.** In a next step, we examined the contribution of the three remaining enzymes to DMSP biosynthesis. 1 mM SMM was incubated with 10 uM of the putative PLP-dependent decarboxylase (SMMCD, Figure 1). This reaction converted all SMM to dimethylsulfoniopropylamine (DMSPamine, **2b**) as inferred by ESI HRMS (m/z calc: 120.0841; obs: 120.0841) and by ^{1}H NMR (Figure 2, Table S2). The same reaction supplemented with 10 uM of the putative aminotransferase (DMSPAAT) and pyruvate, consumed all **2b** and produced one equivalent of alanine (Table S2), suggesting that DMSPAAT transferred the amino group from **2b** to pyruvate. The second expected product dimethylsulfoniopropanal (DMSPaldehyde, **2c**) could not be detected because this compound spontaneously decayed to acrolein by elimination of DMS (Figure S7).[18] In contrast, a reaction containing SMMCD, DMSPAAT together with the aldehyde dehydrogenase (DMSPaldehyde dehydrogenase, DMSPADH) and NAD+, converted 80 % of SMM to DMSP (m/z calc: 135.0472; obs: 135.0474, ^{1}H NMR: Figure 2, Table S2). Finally, a reaction combining these three enzymes with MSMT and SAM was able to catalyze the entire four-step transformation of Met to DMSP (Figure S8). This experiment represents the first *in vitro* reconstitution of DMSP biosynthesis.

Production of DMSP by *S. mobaraensis*. The *in vitro* activity of these four enzymes suggest that *S. mobaraensis* can produce DMSP. To test this expectation, we cultivated *S. mobaraensis* (DMSZ DSM 40847) in liquid culture in a medium containing glucose, yeast-, and malt-extract (GYM-Streptomyces Medium) supplemented with 0, 0.4 or 0.8 M NaCl. Ethanolic extracts from cells grown in 0.8 M indeed contained DMSP as inferred by ^{1}H NMR spectroscopy and by comparison with authentic DMSP (Figure S9). Quantification of the dimethylsulfonium proton signal indicated a cellular DMSP concentration of 0.50 ± 0.06 mM (Figure S10). In contrast, cells grown at low or medium salinity contained less than 50 uM DMSP (Figure S11). Induction of DMSP biosynthesis by osmotic stress is consistent with the idea that this metabolite serves as an osmolyte.[6b, 9] Indeed, most of the fourteen bacterial species that contain close homologs of the DMSP biosynthetic enzymes from *S. mobaraensis* were isolated from saline or hypersaline habitats (Table S1).

Independent origin of bacterial DMSP production. DMSP biosynthesis via SMM (route 2) was also observed in angiosperms such as *W. biflora*[10d] and *Spartina alterniflora* (saltmarsh cordgrass).[10c] However, the following evidence suggests that plant and bacterial DMSP biosynthetic activities may have emerged independently. Since the plant enzymes transforming SMM to DMSP are not known, the descriptions of these pathways are solely based on the structures of detected metabolites. In *W. biflora* **2b** could not be detected as an intermediate, which lead to the proposition that deamination and decarboxylation may be coupled in a single enzymatic step (route 3).[10d] In *S. alterniflora* the conversion of **2b** to **2c** was found to be O_2-dependent,[10c] implicating an oxidase instead of a PLP-dependent transaminase as a catalyst for this step.[19]

A further indication that plant and bacterial DMSP production emerged from different origins comes from the observation that bacterial DMSP biosynthetic enzymes are more related to bacterial enzymes with other functions, rather than to plant enzymes. SMMDC from *S. mobaraensis* shares up to 50 % seqID with putative bacterial diaminopimelic acid decarboxylases. DMSPAAT shares up to 60 % seqID with putative bacterial transaminases, and DMSPADH shares up to 60 % seqID with putative glycine betaine aldehyde dehydrogenases. By contrast, plant proteins are significantly less related to SMMDC (< 30 % seqID), DMSPAAT (< 35 % seqID) or DMSPADH (< 40 % seqID). Although $MSMT_{Str}$ and $MSMT_{WoII}$ catalyze the same reaction they are only distantly related (24% seqID). $MSMT_{Str}$ is much more related to MSMT homologs (> 50 % seqID) from bacteria that contain no discernable homologs of SMMDC and DMSPAAT (< 30% seqID). These sequence relationships suggest that DMSP production in bacteria via route 2 emerged by adaptation of bacterial enzymes from other pathways rather than from gene exchanges with plants.

This finding conflicts with the expectation that secondary metabolite production lines are usually disseminated by horizontal gene transfer.[13] Exchange of ready-to-use genetic instructions between different species generally occurs far more frequently than de novo emergence of new enzyme activity, let alone entire enzyme cascades. However, emergence of DMSP biosynthetic activity may be an unusually simple evolutionary process because of the adaptability of the involved enzyme types. PLP-dependent enzymes are often promiscuous and may adapt substrate and reaction specificities requiring only minimal sequence changes.[20] Aldehyde dehydrogenases too are often characterized by limited substrate specificity not least because bacteria require unspecific aldehyde dehydrogenase activity as a protection against electrophilic stress.[21] Hence, onset of DMSP production and accumulation in an adapting cell may take as little as acquiring an MSMT coding gene by horizontal gene-transfer and downregulation of activities that consume SMM through competing pathways.[22] This simplicity provides a plausible answer for why bacterial and plant DMSP production emerged independently. By extension, we would not be surprised to find additional unrelated DMSP biosynthetic loci as more genome sequences are becoming available.

Conclusions. In this report we described the first *in vitro* reconstitution of DMSP biosynthesis using four enzymes from *S. mobaraensis*. Identification of these enzymes lead to the correct prediction that this actinobacterium is a DMSP producing organism. Although the bacterial pathway follows the same chemical steps as described in plants,[11] limited sequence similarity to plant enzymes suggest that the two pathways emerged through convergent evolution. The broad distribution of MSMT-like enzymes, the adaptability of PLP-dependent enzymes and the promiscuity of aldehyde dehydrogenases may have been key factors making de novo assembly of a DMSP production line in bacteria more efficient than horizontal gene transfer from plants. The discovery of angiosperm-like DMSP biosynthesis in bacteria also lends

further support to the notion that this biosynthetic activity is far more common than previously thought.[9]

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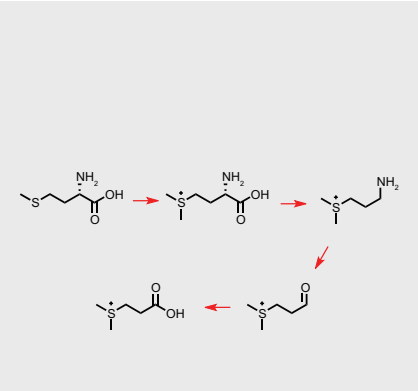

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