Metallomics

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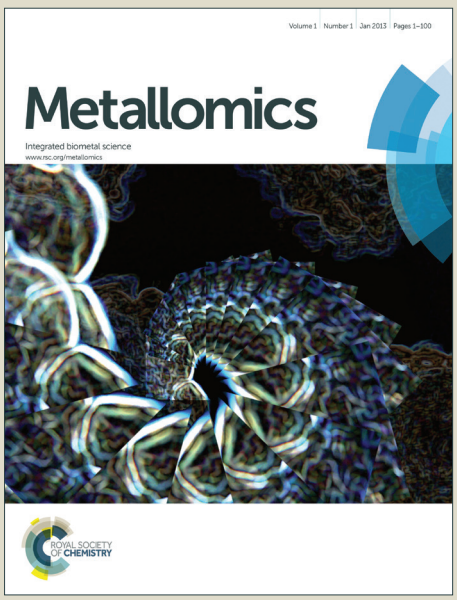

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Perspective: What is known, and not known, about the connections between alkane oxidation and metal uptake in alkanotrophs in the marine environment.

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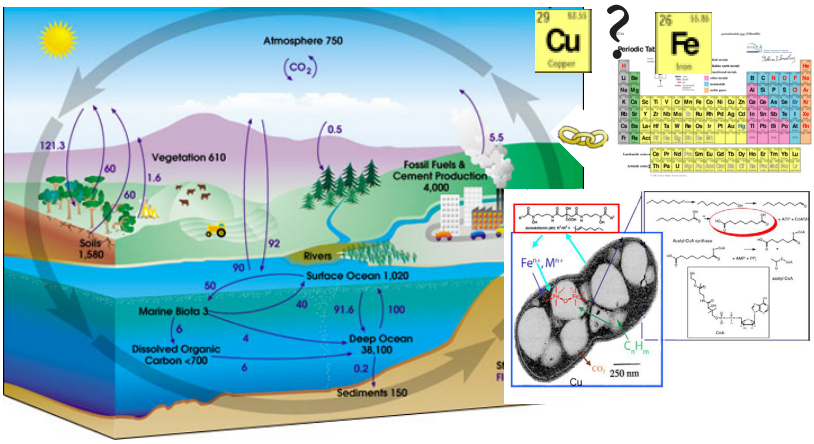

Figure 1. An illustration to highlight both the connections, and our uncertainty as to the their mechanisms, between metal acquisition and alkane utilization in microorganisms that can grow on alkanes.

Article Summary: Should iron and copper be added to the environment to stimulate the natural bioremediation of marine oil spills? The key enzymes that catalyze the oxidation of alkanes require either iron or copper, and the concentration of these ions in seawater is vanishingly low. Nevertheless, the dependence of alkane oxidation activity on external metal concentrations remains unclear. This perspective will summarize what is known about the co-regulation of alkane oxidation and metal acquisition and pose a series of critical questions to which, for the most part, we do not yet have answers. The paucity of answers points

to the need for additional studies to illuminate the cellular biology connecting microbial growth on alkanes to the acquisition of metal ions.

Perspective: The addition of iron in certain regions of the world's oceans to stimulate phytoplankton growth ("iron fertilization") and possibly lower atmospheric CO_2 levels—creates a paradigm for biochemistry in marine environments. Trace elements, especially the all-important first-row transition metals, are essential to the redox-dependent reactions that define biogeochemical cycles. Transition metal ions are used for alkane oxidation by almost all microorganisms that can utilize alkanes as their sole source of carbon and energy[1] —here termed "alkanotrophs" to distinguish them from the larger group of microorganisms that can use any hydrocarbon in this way and are called *hydrocarbonoclastic* (HCB), or the smaller group of microorganisms that live on methane and are called *methanotrophs*. Is there any sort of linkage between alkane oxidation and metal acquisition in these organisms? Is the *regulation* of these processes linked in any way?

This perspective will examine this question and pose a series of related sub-questions. We begin by examining methane oxidation, where a clear connection between methane oxidation and iron and copper uptake exists. Key questions, which we pose, remain unanswered even in this case, which represents the best-

[1] A summary of the enzymes used for aerobic oxidation of alkanes along with information about their active site composition is included in the supplemental material.

understood example. An examination of what is known about the co-regulation of metal uptake and the oxidation of longer chain alkanes yields more questions and fewer answers. Several transcriptomic and proteomic studies have been conducted on alkanotrophs to identify the proteins differentially expressed when bacteria are grown on longer chain alkanes vs. other carbon sources. However the evidence from these publications, reviewed briefly in this perspective, neither confirms nor rules out a regulatory connection between growth on longer chain alkanes and metal acquisition. We pose critical questions about the potential co-regulation of metal acquisition and alkane oxidation and point to testable hypotheses, which in the spirit of a *Metallomics* perspective, draws the community's attention to areas where further developments are urgently needed.

The "copper switch" between soluble and particulate methane monooxygenase (MMO) is the classic case of metal-dependent expression of alkane oxidizing enzymes, and its existence raises the question of whether this connection extends to other alkanes. In organisms that can express both the iron-containing sMMO and the copper-containing pMMO, sMMO is expressed when the concentration of copper is below 0.8 micromolar,[1, 2] whereas large amounts of pMMO are produced in extensive intracytoplasmic membranes at copper concentrations of about 4 micromolar. Ultimately, pMMO can comprise up to 20% of the total cellular proteins in methanotrophs.[3]

While the link between copper concentrations and MMO expression is well established, the details of how this switch works are not well understood. What is known is that in the presence of Cu or a small Cu-chelating natural product known

as methanobactin (Mbn), pMMO is the only methane-oxidizing protein expressed.[3, 4] It is also known that sMMO regulation is copper-dependent. The sMMO operon is significantly downregulated—by several orders of magnitude—when copper is added.[5, 6] pMMO regulation appears to be partially copper-dependent.[6] There is some evidence for σ^{70}-mediated background constitutive expression.[6] There is *also* evidence for some up-regulation of transcription in the presence of copper,[5] but the amount of observable copper-stimulated increase in transcription has varied among experiments, techniques, and research groups. Post-transcriptional regulation has also been proposed to occur.[6] It is not known what regulators are involved in the link between iron and copper and MMOs, or whether there is any direct link to iron homeostasis mechanisms. No studies have identified any external binding sites for the regulator in the sMMO operon, a σ^{54}–transcriptional activator known as mmoR.[7] Moreover, this regulator has not been demonstrated to bind copper, and no other interactions between the sMMO promoter regions and a copper-binding regulator have been identified, so the trigger point of the copper switch remains a mystery.

Thus, although the copper-dependent reciprocal expression of pMMO and sMMO is well documented, the literature remains surprisingly unclear on the regulatory mechanisms behind this process. A few "omics" studies have been carried out on methanotrophs, but they have been focused mostly on variables other than copper,[8] or have been limited in scope.[9, 10] Moreover, the broader studies that were carried out were done on *Methylococcus capsulatus* (Bath), a species that does not produce high-affinity Mbns resembling those characterized previously.[11 4]

The literature is even less clear on whether there is a connection between growth on longer chain alkanes and metal acquisition that is in any way independent of the metal acquisition response microorganisms are known to have in metal-limited environments. In 2011, Sabirova *et al.* published a transcriptional study of the best-studied alkanotroph, *Alcanivorax borkumensis*, comparing gene expression when the organism was grown on pyruvate vs. hexadecane.[12] The authors show an increase in iron uptake genes, including an Fe^{3+}-hydroxamate ABC transporter protein and a ferripyoverdine receptor precursor (FpvB)—and also the genes for two alkane monooxygenases (*alkB1* and *alkB2)* and two flavin monooxygenases—when cells are grown on hexadecane. Intriguingly they also see an increase in a copper binding protein, CopC, and a copper-translocating P-type ATPase. By contrast, another transcriptomic study of *Alcanivorax borkumensis* and its response to alkanes and toxic organic compounds showed no evidence that metal uptake genes are upregulated when cells are grown on alkanes, although the studies were done in mineral medium that was not iron-limited. [13]

A tantalizing result comes from a recent report that provides the genome sequence as well as a functional analysis of the oil-degrading bacterium *Oleispira antarctica*.[14] Siderophore production increases when *O. antarctica* cells are grown on iron-limited material and *n*-tetradecane, although production levels off as the number of cells continues to increase. However, the authors did not test whether siderophore levels would have gone up identically had the cells been grown on another carbon source under the same iron-limited conditions.

Results from proteomic studies are also mixed. The first proteomics paper on

a non-methanotroph alkanotroph showed that some transporter proteins are upregulated when *Alcanivorax borkumensis* is grown on hexadecane.[15] These transporters include OprG, an ABC transporter that is thought to be a low affinity transporter of iron. A recent proteomics paper from Grimaud *et al.*[16] reported no increase in AlkB when *Marinobacter hydrocarbonoclasticus* SP17 cells are grown on alkanes. This has been attributed to the known difficulty in extracting and detecting integral membrane proteins with 2D electrophoresis.[17] However, they did find that iron uptake and transport proteins, including FhuE, FbpA and CirA, were upregulated when *Marinobacter* cells were grown on hexadecane and formed biofilms.[16]

In some non-marine organisms, suggestive links between hydrocarbon oxidation and iron requirements have been observed. *E coli* and *P. oleovorans* (*P. putida*), when overexpressing AlkB, have been shown to have an increased cellular demand for iron.[18] *Pseudomonas* strains that oxidize toluene using an AlkB-like enzyme, XylM, have shown decreased toluene degradation in iron-limited continuous culture experiments.[19] Finally, iron stimulates surfactant production in *Bacillus subtilis,*[20] and similar mechanisms have been hypothesized for facilitating hydrocarbon oxidation in the presence of added iron in pseudomonads.[21]

Is there evidence for a link between trace metal concentrations and alkane oxidation in the environment? In mesocosm studies in Alaska, Exxon scientists added iron along with nitrogen and phosphorus to stimulate growth of naturally occurring alkane-oxidizing bacteria and found that the addition of fertilizers increased oil metabolism.[22] The lead author of the study speculates that iron

addition was unnecessary and that N and P would have been sufficient to stimulate growth because hydrocarbons are particularly deficient in these key elements.[23] Peter Golyshin, who performed the first omics-based studies on HCB, frequently studies enrichment cultures of organisms growing on alkanes and observes significant growth stimulation when the medium is supplemented with N and P to reach common textbook proportion of approximately 50:14:3 (C/N/P)—but notes that iron addition does not affect cell density.[24] However, some HCB rely on siderophore piracy to acquire iron and so may not respond to iron unless it is chelated.[25,26]

Finally, is there evidence for a physical connection in the marine environment between siderophores and alkanes? Siderophores from marine organisms that degrade oil are well characterized and notably hydrophobic.[27] Some organisms are known to adhere to oil droplets.[28] Do hydrophobic siderophores adhere to oil droplets as well, and perhaps co-localize iron, alkanes and bacteria in oil-rich environments?

What conclusions can be drawn? None, as of yet. Two working hypotheses could be tested, however. First, growth on liquid alkanes does not stimulate significant iron uptake that is independent of iron-uptake stimulated by low concentrations of iron in the environment. Second, the lack of abundance of other nutrients such as nitrogen and phosphorus in native alkanotroph environments is a greater limiting factor than iron deficiency.

What other hypotheses can we offer? Methane oxidation, or at least the copper-dependent pMMO system, is unique. pMMO comprises as much as 20% of

the total protein in a bacterium growing on methane and each pMMO trimer contains 6-9 coppers, so the total amount of copper required for a methanotroph growing on methane is high. In contrast, AlkB accounts for approximately 1% of total cellular proteins, and each AlkB contains only two irons. Marine organisms are already poised to synthesize siderophores and acquire iron in low iron environments because they need iron for a host of cellular functions. Despite some observed connections between iron acquisition and hydrocarbon oxidation, it may be that the additional iron demand imposed by production of catalytically active iron-dependent alkane monooxygenases is not significant enough to establish an iron acquisition process that is co-regulated with growth on alkanes.

The question of differential metal-dependent regulation during expression of different alkane monooxygenases leads naturally into the question of broader alkane-dependent regulation. Many alkanotrophs are capable of growing on a few other substrates in addition to alkanes; thus, they must have a dynamic regulatory system that allows them to conditionally express the proteins required for growth on alkanes when those compounds are present. Alkanes can be abundant in the marine environment, but their concentrations can also be highly variable, so an evolutionary strategy that enables an organism to utilize other sources of energy might be important. While many methanotrophs grow only on methane, some facultative methanotrophs do exist (*Beijerinckaceae*, *Methylocapsa*, *Methylocella* and possibly *Crenothrix* species).[2] Hydrocarbonoclastic bacteria have been found associated with dinoflagellates,[29] leading to the hypothesis that they live in communities with algae, protists, and other photosynthetic organisms and, in the

absence of alkanes, feed on their fatty acids and lipids. When these organisms switch between growth on a non-alkane substrate and growth on an alkane, is activation of metal acquisition machinery necessary, or is the existing metal quota sufficient to metallate new alkane-oxidizing proteins? If stimulation of additional metal uptake is unnecessary, is there a mechanism for redistributing the metals already present to the newly synthesized alkane oxidizing enzymes?

Most alkanotrophs contain multiple alkane-oxidizing enzymes, a redundancy that is not fully understood. In some cases, different alkane oxidizing enzymes are used for alkanes of different chain length; an organism would presumably synthesize the alkane-oxidizing enzyme needed for a particular range of alkanes when presented with those alkanes. As straightforward as it sounds, even this would require a sophisticated regulatory mechanism: How are alkane uptake and oxidation linked? Are there different receptors for different chain-length alkanes? Does receptor specificity overlap with the specificity of particular alkane-oxidizing enzymes? More transcriptomic and proteomic studies on a broader range of alkanotrophs and growth conditions could answer these questions.

There are also redundant alkane-oxidizing enzymes that appear to catalyze the oxidation of alkanes of the same chain length. Medium chain length alkanes can be oxidized by both a soluble cytochrome P450 (CYP) with a single iron ion at its active site or a particulate alkane hydroxylase (AlkB) with two iron ions per monomer in its active site. Perhaps, as some studies have suggested, CYP is constitutively expressed while AlkB is induced when microorganism are exposed to most medium chain length alkanes (although one paper reports a clear increase in

CYP levels in response to very specific alkanes[30]). Perhaps soluble alkane oxidizing enzymes are "convenient"—easy to keep around in case an alkane comes by that could be oxidized. The presence of both sMMO and pMMO in some methanotrophs may reflect a similar paradigm. We postulate that there must be some advantage to using membrane-spanning proteins to oxidize alkanes—perhaps simply the greater solubility and stability of alkanes in the hydrophobic environment of a membrane—although developing a full understanding the nature of that advantage awaits further investigation.

In the future, a key approach to deciphering the connection between alkane utilization and metal acquisition in alkanotrophs will be RNAseq experiments using several different alkanotroph strains growing on both alkanes and other substrates under a range of iron concentrations, both aqueous and siderophore-bound. These experiments could reveal whether conditions exist under which growth on alkanes induces more siderophore production, or more iron uptake proteins, than growth on a non-alkane substrate at the same iron concentrations and cell density. Similar experiments could be used to address another question, namely, whether growth on longer-chain alkanes that require a flavin-based enzyme results in less stimulation of iron acquisition and uptake genes than growth on medium-chain alkanes that require iron enzymes for alkane activation.

The complexity of the cellular biology of alkane oxidation is underscored by a recent paper in PLOS One.[30] In this paper, the genome of the moderate halophile *Amycolicicoccus subflavus* DQS3-9A1^T was found to include genes for three AlkBs, two CYPS, one LadA, and a cluster of genes related to propane monooxygenase.

Expression of all genes related to alkane oxidation was stimulated by growth on at least one alkane, with the expression of some genes, like the one associated with propane monooxygenase, showing a sharp increase in response to only one alkane (propane in this case), while others, including all three *alkB*s, showed an increase in response to essentially all alkanes tested Stimulation of the expression and production of the correct alkane oxidizing enzymes in an organism like this must require a complex set of regulatory interactions.

In summary, there is still no definitive evidence that alkane oxidation in coastal environments is limited by the availability of metal ions. There are, however, examples of clear links between metal uptake and alkane oxidation, but the extent of the dependence of alkane oxidation on metal uptake in alkanotrophs remains unclear. As transcriptomics and proteomics experiments become more common, we will be able to probe the responses of alkanotrophs to a range of alkane substrates and metals in conditions that mimic the open ocean. Such data will address the many remaining questions surrounding the alkane- and metal-dependent regulation of alkane oxidizing enzymes and the acquisition, uptake, and cellular trafficking of both metal ions and alkanes.

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