C_1-Transfer Modules: from Genomics to Ecology

Differences among C_1-transfer metabolic modules provide insights about microbes with different environmental functions

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The metabolism of C_1 compounds, those such as methane, methanol, methylated amines and halogenated methanes that contain no carbon-carbon bonds, is an important part of the global carbon cycle. In aerobic environments across a range of oxygen tensions, salinity readings, pH values, and temperatures, the methylotrophic bacteria are the major players in C_1 cycling.

Methylotrophy also is widespread across bacterial groups and is found in alpha-, beta-, and gamma-proteobacteria and in gram-positive bacteria. Many representatives of methylotrophic genera, such as *Methylobacterium extorquens, Methylococcus capsulatus, Paracoccus denitrificans*, and *Methylobacillus flagellatus*, are readily cultivated in the laboratory, lending themselves to biochemical, physiological, and genetic studies. Whether these and other cultivated methylotrophs fully reflect the makeup of organisms in the environment and where they fit into the evolutionary history of methylotrophy are among the central unanswered questions facing researchers who study these organisms. Recent efforts to use environmental genomics as a means for analyzing methylotrophs are providing valuable insights into C_1-cycling populations in natural habitats.

- Methylotrophic bacteria are the major players in globally cycling C_1 compounds such as methane, methanol, methylated amines and halogenated methanes.
- Methylotrophs carry specific metabolic modules for oxidizing C_1 compounds, but some of these modules also occur among bacteria that are not methyltrophs, and likely are used for scavenging and detoxifying purposes.
- Finding culture-independent H_4MPT-linked C_1 oxidizing capacities in both Archaea and Bacteria suggests this pathway was present in the universal common ancestor before these Domains separated.

Modular Nature of Methylotrophy

Complex metabolic networks consist of series of discrete functional units, or modules, each devoted to specific metabolic activities. Simply put, a module is a set of genes and encoded enzymes involved in conducting a specific metabolic function. Genes in a module may encode enzymes that catalyze one or a sequence of several reactions. Removing or mutating any genetic component in a module would have a similar effect on phenotype.

An important attribute of a module is its replaceability. Thus an alternative module with a similar function (nonorthologous substitution) can replace an existing module.

Methylotrophy consists of a set of functional modules as determined by mutational studies in model methylotrophs and as deduced from genomic sequences of methylotrophs (Fig. 1). Specific modules oxidize C_1 compounds, including methane monooxygenase (MMO), methanol dehydrogenase, methylamine dehydrogenase, corrinoid-dependent methyltransferase, or methanesulfonic acid monooxygenase. With few exceptions, these enzyme-catalyzed reactions produce formaldehyde. Ad-

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FIGURE 1

Examples of methylotrophy functional modules, based on genomic analyses. Primary oxidation modules are in green; formaldehyde oxidation modules are in red; formate oxidation modules are in purple, and C_1 assimilatory modules are in yellow.

ditional modules, such as the serine cycle and the ribulose monophosphate (RuMP) cycle, are involved in assimilating C_1 formaldehyde units. The rest of formaldehyde being produced is oxidized further to formate.

One of the most widely distributed formaldehyde oxidation modules is the one involving tetrahydromethanopterin (H_4MPT) as a cofactor. However, it is not found in some autotrophic methylotrophs, such as *P. denitrificans*, where a module linked to glutathione (GSH) operates instead. Some methylotrophs, such as *M. flagellatus*, rely on the RuMP cycle for formaldehyde oxidation, while the H_4MPT pathway fulfills an auxiliary function. Still other methylotrophs possess a nicotinamide adenine dinucleotide (NAD)-linked formaldehyde dehydrogenase; however, the function of this enzyme in methylotrophy has not yet been tested by mutation.

Formate resulting from oxidation of formaldehdye is further oxidized to CO_2 via specific formate oxidation modules (formate dehydrogenases), a variety of which are found in methyltorophs. Some methylotrophs (methylotrophic autotrophs) assimilate C_1 units exclusively at the level of CO_2, via the Calvin-Benson-Bassham (CBB) cycle, while others possess modules for assimilating both formaldehyde and CO_2. Many known methylotrophs possess multiple modules for specific steps of methylotrophy (Fig. 1).

In some cases, these multiple modules appear to be redundant. For example, each of the membrane-bound MMO modules may be removed from *M. capsulatus* with little effect on its growth, according to Sergey Stolyar and colleagues of the University of Washington, Seattle. In the case of *M. extorquens*, mutating all three formate dehydrogenase modules did not affect the ability of such mutants to grow on C_1 compounds other than formate, and any one of the three could grow on formate. All three C_1 assimilatory pathways encoded in the genome of *M. capsulatus* are expressed during growth on methane, according to a genome-based proteomic analysis by Wei-Chun Kao and colleagues of National Taiwan University.

Theoretically, to survive as a methylotroph, an organism requires at least one functional module to oxidize C_1 substrates, to oxidize and detoxify formaldehyde, and to assimilate C_1. However, for some less-studied methylotrophs, not all these modules are recognizable at the

A Biologist and Systems Engineer, Lidstrom Sees Bacteria as Engineering Prototypes

When Mary Lidstrom looks at a bacterium, she sees a sophisticated engineering system with mechanisms that can be applied to what humans build. A biologist by training, she is also a systems engineer who values using an engineering approach to study complex biological systems. Today, among other things, she teaches microbiology to engineers.

"We . . . teach biology to engineers differently than it is taught to biologists," she says. "It's a function-based approach, with the idea of nature as the designer and evolution as the design tool. That's real engineering. And that's the way biology should be taught—start with how it works, then talk about the parts."

Lidstrom encourages others involved in the UW engineering-life sciences program to relate design and function issues to biology. If scientists and engineers can understand how nature does some particular task, they may figure out how to adapt those principles to other tasks, she says. For instance, human skin has a remarkable ability to repair itself, while the shell of the abalone "is made up of minerals and proteins, and is several times stronger than it should be, based on its components."

For nearly three decades, Lidstrom has studied how metabolism is integrated within bacterial cells. She focuses specifically on bacteria that grow on one-carbon compounds—methylotrophs. "The production, interconversion, and transfer of C_1 units is an important basic metabolic system in all of biology, with both environmental and biomedical implications," she says. These bacteria can grow on methane, methanol, and methylated amines. One long-term goal is to develop environmentally sound and economically viable alternatives to current chemical production and cleanup strategies, she says.

Besides teaching and doing research in microbiology, Lidstrom has been associate dean in engineering at the University of Washington (UW) since 1997. In this role, she has been a major force behind integrating life sciences into the engineering program. She first became involved with academic administration at the California Institute of Technology, where she taught environmental engineering science from 1987–1996. She ran a research center and training grant there, and later became vice-chair of the faculty. "These experiences taught me much about how to work with teams and build consensus, beyond the confines of the research laboratory," she says.

Those experiences doubtless continue to come in handy when dealing with current pressures. "Facing challenges such as diminishing state and federal funding, and an increasingly hostile regulatory environment is offset each time one of our junior faculty gets their first grant, graduates their first Ph.D. student, or wins a teaching award," she says. Being an associate dean allows her "to continue working with students, teaching, and carrying out research, while also being involved in helping the next generation of faculty leaders succeed."

Lidstrom also oversees an outreach program in genomics. The program recruits high school students into doing genomics research after their junior year and lets them continue such work well into graduate school. The program actively seeks students of color and those from disadvantaged backgrounds. In addition, she has been a mentor to countless undergraduates who have done research in her lab over the years. "With each of them, I help repay the strong beginnings I had so long ago," she says.

Lidstrom grew up in Prineville, Ore., a small town in the center of the state, on a 1,000-acre cattle ranch, the youngest of four children. This experience "put me in touch with biology on a daily basis. I was always fascinated with how biology worked." She became hooked on biological mechanisms in 7th-grade science, "when our teacher used elementary chemistry to explain diurnal cycles of turgor pressure in plants."

Later, as a high school senior, she met Leo Parks, a professor of microbiology at Oregon State University (OSU). "Leo convinced me that if I wanted to study biological mechanisms, microbiology was the right major for me," she recalls. She took his advice, studied microbiology at OSU, graduated in 1973, and then moved to the University of Wisconsin where she received a M.S. in bacteriology in 1975 and a Ph.D. two years later. She is married to Charles Chavkin, a UW pharmacology professor, and they have two children, 13 and 16.

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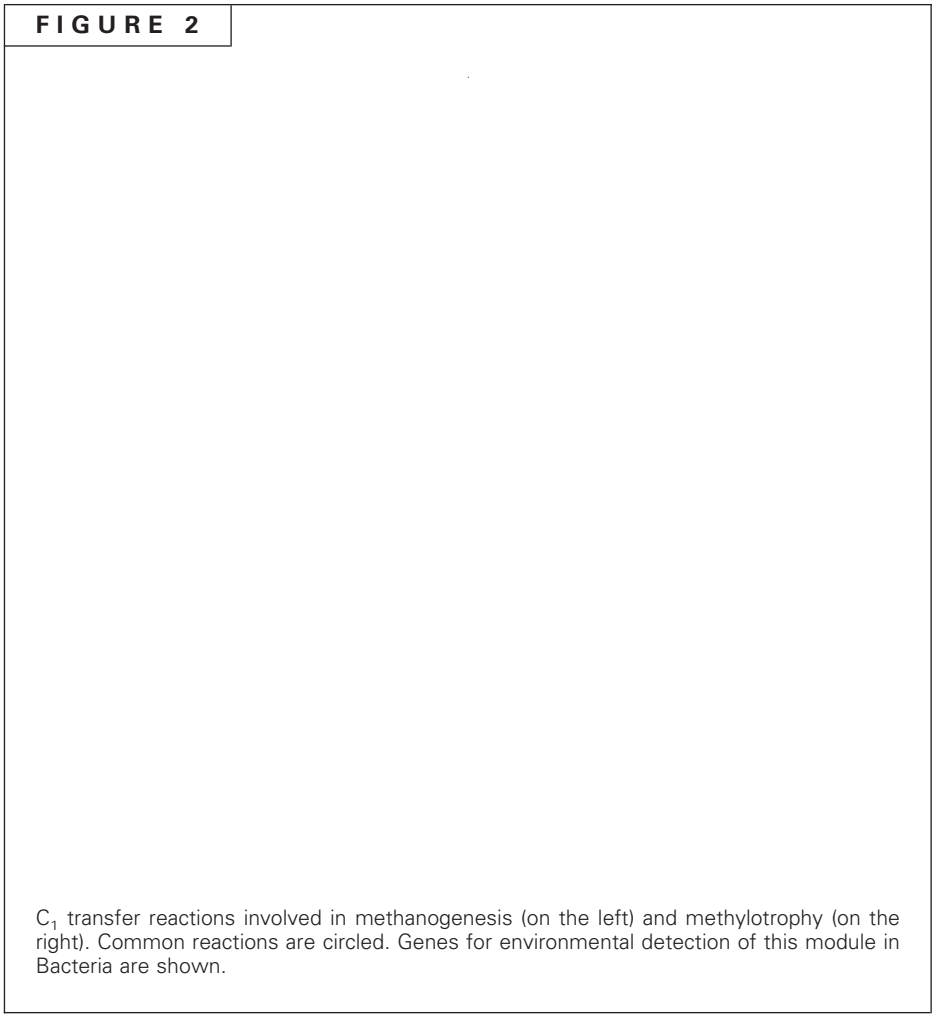
FIGURE 2



C_1 transfer reactions involved in methanogenesis (on the left) and methylotrophy (on the right). Common reactions are circled. Genes for environmental detection of this module in Bacteria are shown.

genomic level. For example, the genome of *Methylibium petrolephilum* encodes no recognizable methanol dehydrogenase.

Meanwhile, methylotrophy modules sometimes are found in genomes of organisms not known for methylotrophic ability—for example, *Burkholderia xenovorans* (Fig. 1). These modules might metabolize formaldehyde that is produced as by-product of biodegradative processes. For instance, three formaldehyde oxidation functional modules in *B. xenovorans* are redundant when metabolizing formaldehyde that is produced when the organism oxidizes choline. Among them, the NAD-linked formaldehyde dehydrogenase plays a major role while the H_4MPT-linked pathway plays a minor role, according to Chris Marx and others at the University of Washington. Similarly, both the GSH-linked and the H_4MPT-linked modules for formaldehyde oxidation, as well as the CBB module for CO_2 assimilation, are up-regulated when this same organism enters late-stationary growth on polychlorinated biphenyl, according to Vincent Denef and colleagues of the Center for Microbial Ecology in East Lansing, Mich. They suggest that the organism may be switching to a scavenging mode, which would be consistent with what *B. xenovorans* does when metabolizing C_1 compounds in the rhizosphere, where it likely encounters many methylated aromatic compounds.

The H_4MPT-Linked Formaldehyde Oxidation Module

The formaldehyde oxidation module that is linked to H_4MPT is of special interest for tracking the evolutionary history of methylotrophy and because of similarities with methanogenesis reactions and genes in *Archaea* (Fig. 2).

When this oxidative pathway was discovered in *M. extorquens*, some researchers quickly surmised that methylotrophic bacteria acquired this set of genes via lateral transfer from a methanogenic archaeon. However, Thomas Cavalier-Smith of the University of Oxford instead proposed that the pathway appeared first in methylotrophic bacteria and later transferred into *Archaea*, in which it enables methanogenesis.

To address evolutionary questions regarding the occurrence and distribution of H_4MPT-linked formaldehyde oxidative capacity among microbes, we (the authors of this paper) developed polymerase chain reaction (PCR) primers to detect this pathway in specimens obtained from various environments.

Before developing those primers, we compiled databases of bacterial genes involved in H_4MPT-linked reactions—identifying corresponding chromosomal fragments in large insert fosmid libraries of selected methylotrophic species and also searching genomic sequences for those genes. We were fortunate to include data not only from proteobacterial genomes, such as those of *M. capsulatus* or *B. xenovorans*, but

FIGURE 3

Strategies used for linking genomic knowledge with ecological function.

also from genomes representing the deeply-diverging division of bacteria, *Planctomycetes*, including *Rhodopirellula baltica*, *Gemmata obscuriglobus*, and *Gemmata* sp. Wa1–1.

The planctomycete sequences diverge significantly from both proteobacterial and archaeal sequences. The importance of this observation is twofold. First, the phylogenetic analyses involving proteobacterial, archaeal, and planctomycete sequences argue against early lateral transfers between *Archaea* and *Bacteria*. Instead, they are more consistent with either the presence of reactions linked to H_4MPT in the last universal common ancestor or in their emergence in *Planctomycetes*. Second, our analysis of the planctomycete sequences provides the first clues into the degree of divergence of the genes and enzymes in question within the Bacterial domain of life, both pointing to a long evolution of H_4MPT-linked reactions within this kingdom and to the challenges of developing environmental tools targeting these genes.

From a total of 16 genes included in comparative analyses, only 4 were sufficiently conserved to design degenerate PCR primers (*mch, mtdB, fae* and *fhcD*; see Fig. 2) for use in probing environmental specimens.

C_1 Transfer Genes in Lake Washington

We used these primers to probe specimens obtained at a Lake Washington site to test for C_1-transfer genes. The top centimeter of the Lake Washington sediment is characterized by steep gradients of methane and oxygen, supporting a thriving community of aerobic methanotrophs that is dominated by gamma-proteobacterial (type I) methanotrophs, with about 10-fold lower population of alpha-proteobacterial (type II) methanotrophs, according to Andria Costello, Ann Auman, and their colleagues from the University of Washington.

Although we had no information about C_1 users other than methanotrophs in these Lake Washington sediments, we soon found that our *fae, mtdB, mch,* and *fhcD* primers successfully PCR-amplified fragments from these sites whose sequences corresponded to not only those of gamma- and alpha-proteobacterial methanotrophs, but also the ones clustering with the sequences of nonmethanotrophic methylotrophs, suggesting cycling of C_1 compounds other than methane in these sediments.

Meanwhile, most of the amplified sequences did not belong to known methylotroph groups, pointing toward novel populations with C_1-ox-

idizing capacities. Some of these sequences appear to be alpha- or gamma-proteobacterial sequences, while others corresponded to not-yet-cultured methylotrophic species. Some in this latter group appear to belong to beta-proteobacteria and are closely related to *B. xenovorans* and *M. petroleophilum*, suggesting a function either in oxiding methanol or in scavenging formaldehyde from decaying organic compounds. Many sequences in the *fae* and *fhcD* libraries and some in the *mtdB* library diverge significantly from proteobacteria, planctomycetes, or archaea, and thus could represent novel phyla capable of H_4MPT-linked C_1 transfers.

To further characterize C_1-transfer gene distributions within the microbial community in Lake Washington sediment, we analyzed large-insert metagenomic libraries. By comparing the number of *fae* and *fhcD* genes to the number of16S rRNA genes in these libraries, we estimate that an unexpectedly broad 20–25% of microbial genomes present in this environment contain H_4MPT-linked transfer genes.

Further, a large percentage of the *fae* and *fhcD* genes in the metagenome belong to novel, unaffiliated groups of sequences identified in the PCR-based libraries. Our analysis of clones in the metagenomic library suggests that divergent, nonaffiliated *fae, fhcD,* and *mtdB*-like genes were parts of clusters containing genes homologous to those encoding H_4MPT-linked C_1 transfer enzymes, further supporting their affiliation with yet unknown and uncultured microbial phyla possessing the C_1-oxidative capacity.

When we employed mRNA-based fluorescent in situ hybridization (FISH) analysis, we found that some of these genes as well as planctomycete-like genes are expressed in situ. Based on the abundance and expression of these novel genes, we believe that these organisms are likely involved in important environmental processes, such as degrading complex compounds to yield products such as formaldehyde. When we supplied these organisms with C_1 substrates, such as formaldehyde and humic acids, in microcosms, then used PCR to amplify *fhcD* genes, some of the novel sequences we analyzed were enriched only in the presence of formaldehyde or humic acids, and not in the presence of C_1 substrates (methane, methanol) that support growth of methylotrophs. These results further suggest that the organisms possessing these sequences are only involved in metabolism of formaldehyde or more oxidized C_1 compounds.

C_1 Transfer genes in the Sargasso Sea

Craig Venter and colleagues at the J. Craig Venter Institute (formerly the Institute for Biological Energy Alternatives) in Rockville, Md., have recently generated a genomic database of microbes inhabiting the Sargasso Sea. Two types of methylotrophs are readily isolated from this environment, the methanotrophs of the genus *Methylomicrobium* and the methylamine utilizers of the genus *Methylophaga*, according to John Sieburth and colleagues of the University of Rhode Island.

When we analyzed the Sargasso Sea metagenome for sequences belonging to these or other methylotrophs, we employed known C_1 primary oxidation gene sequences, such as *pmoA, mmoX, mxaF, cmuA*, *msmA*, and *msmB*, as well as sequences of 17 genes involved in the H_4MPT-linked formaldehyde oxidation. Among the primary C_1 oxidation genes, we detected only those genes similar to *msmA* and *msmB* on singleton reads. However, we also found a number of genes for H_4MPT-linked C_1 transfer reactions in the database, and some of these were parts of assembled scaffolds, pointing to relative abundance of the organisms possessing them.

The sequences uncovered from microorganisms in the Sargasso Sea have no matches in databases that we built for known proteobacterial and planctomycete genes or for any of the novel sequences uncovered in Lake Washington. These data suggest that the Sargasso Sea sequences belong to a novel, yet-uncultivated group of organisms, and that the C_1 transfer module is unlikely associated with primary C_1 substrate oxidation. We believe it more likely to be part of a different ecological process—for example, scavenging formaldehyde derived from dissolved organic carbon compounds.

Energy Generating versus Detoxifying Function of the H_4MPT-Linked Pathway

The H_4MPT-linked pathway fulfills two distinct roles in methylotrophs such as *M. extorquens*, according to experiments by our group at the University of Washington in collaboration with colleagues from the Max Plank Institute in Germany and the National Center for Scientific

Research (CNRS) in France. First, it generates metabolic energy in the form of NADH (catalyzed by MtdB, Fig. 2) and, second, it detoxifies formaldehyde. However, Julia Vorholt of CNRS with colleagues from the University of Washington recently discovered that the novel, divergent *mtdB*-like sequences detected in the Lake Washington metagenome and also the *mtdB*-like sequences in the planctomycete genomes encode an enzyme, designated MtdC, that differs from MtdB in methylotrophs. The C_1 transfer pathway employing MtdC instead of MtdB is predicted to produce NADPH instead of NADH. Thus, this NADP-specificity of the MtdC pathway in planctomycetes and in the uncultivated species from Lake Washington suggests that the pathway is not so important for generating energy but may be important for detoxifying formaldehyde. Alternatively, the NADP-linked C_1 transfer pathway may be involved in producing reduced equivalents for biosynthetic reactions within these cells. In either case, the organisms possessing MtdC would not be expected to grow on traditional C_1 compounds such as methane or methanol because of energetic constraints.

The Antiquity of the C_1 Transfer Pathway

Culture-independent detection of H_4MPT-linked C_1 oxidizing capacities in microbial specimens from various environments has dramatically changed our views on the occurrence, divergence, environmental distribution, and evolution of these reactions. Surely their presence in the deeply branching methanogenic, sulfate reducing, and methane-oxidizing archaea, as well as in deeply branching phyla within *Bacteria* points to the ancient origins of the pathway.

Based on the divergence of the genes involved, we think that that the formaldehyde-oxidizing capacity likely evolved early and was present in the universal common ancestor, before *Bacteria* and *Archaea* separated. Moreover, before methylotrophy or methanogenesis emerged, the early C_1 transfer module might have been used primarily for detoxifying formaldehyde, which is thought to have been abundant on the early Earth. Such a prototype module would have provided a selective advantage for organisms carrying those genes, even without generating energy. Later, additional fitness came from the energy drawn from these reactions.

We further speculate that selective pressure for those energy functions led to the emergence of two (nonhomologous) methylene-H_4MPT dehydrogenases in *Archaea* (Mtd and Hmd) and three methylene-H_4MPT dehydrogenases in *Bacteria* (MtdA, MtdB, and MtdC). The latter are not related to the archaeal enzymes but are related to each other. Such energy-generating C_1 transfer modules could also have provided building blocks for both methanogenesis and methane oxidation.

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