Specialized Metabolites from Methylotrophic Proteobacteria

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Abstract

Biosynthesized small molecules known as specialized metabolites often have valuable applications in fields such as medicine and agriculture. Consequently, there is always a demand for novel specialized metabolites and an understanding of their bioactivity. Methylotrophs are an underexplored metabolic group of bacteria that have several growth features that make them enticing in terms of specialized metabolite discovery, characterization, and production from cheap feedstocks such as methanol and methane gas. This chapter will examine the predicted biosynthetic potential of these organisms and review some of the specialized metabolites they produce that have been characterized so far.

Introduction

Specialized metabolites, also known as secondary metabolites or natural products, form the basis of many bioactive compounds essential to modern medicine and agriculture (Demain and Sanchez, 2009; Cantrell *et al.*, 2012; Newman and Cragg, 2016). This makes intuitive sense because production of these compounds is genetically encoded in biosynthetic gene clusters (BGCs) and therefore the resulting structures have had millennia to evolve their roles. The great value of these molecules in manipulating biological systems means there is a constant demand for new sources of these compounds and strategies for determining their biological functions.

The explosion in bacterial genome sequences available in public databases as well as the availability of bioinformatics tools for analysing them has revealed that many bacterial species are potentially untapped sources for new molecules (Cimermancic *et al.*, 2014). This includes organisms beyond those traditionally relied upon for natural product discovery, and recent studies have shown that examining the biosynthetic potential of new species indeed reveals new classes of compounds (Pidot *et al.*, 2014; Pye *et al.*, 2017). This strategy is complementary to synthetic biology approaches focused on activating BGCs that are not normally expressed under laboratory conditions in strains traditionally used for natural product discovery, such as *Streptomyces* (Rutledge and Challis, 2015).

This chapter will focus on the methylotrophic Proteobacteria, which use reduced carbon compounds with no carbon–carbon bonds as their sole sources of carbon and energy. Specifically, the Proteobacteria are some of the most well-studied organisms within this metabolic group (Chistoserdova *et al.*, 2009). While several molecules have been isolated from methylotrophic Proteobacteria that have had an impact on the field of natural products (Kenney and Rosenzweig, 2018a; Khmelenina *et al.*, 2015), our understanding of the specialized metabolism of these organisms is still in its infancy.

Methylotrophic bacteria as an underexplored resource for specialized metabolite discovery

Methylotrophic bacteria are an attractive source for the discovery of new specialized metabolites for several reasons.

1 Obligate methylotrophs, including many species of methane-oxidizing bacteria, may have been overlooked during traditional 'grind and find' searches for new compounds that focused on the isolation of specific genera like *Streptomyces* using rich media.
2 The lack of complex carbon substrate in methylotroph growth medium can aid in the rapid analysis of new compounds produced by a culture without laborious prefractionation procedures, which may also help in high throughput screening approaches.
3 The chemically defined nature of the growth medium and simplicity of the carbon source also allows substrates with heavy labels such as $^{15}NO_3$ and $^{13}CH_4$ or $^{13}CH_3OH$ to be used for minimal cost, which can aid in structural elucidation using mass spectrometry and NMR.
4 If a molecule with attractive properties is identified, its methylotroph producer can be grown at scale using a feedstock such as methane or methanol, both of which have recently re-emerged as substrates of interest in industrial microbiology (Schrader *et al*., 2009; Henard and Guarnieri, 2018).

It is therefore worthwhile to discover more molecules produced by this ecologically important group of bacteria.

Genome sequencing efforts supported by entities including the Joint Genome Institute (JGI) and Organization for Methanotroph Genome Analysis (OMeGA) have enabled a detailed examination of the predicted biosynthetic potential of many methylotrophic species (Fig. 12.1). Amongst the strains analysed here, more BGCs are generally predicted in methanotroph genomes compared with the genomes of non-methanotrophic methylotrophs. Overall the Alphaproteobacteria, including members of the *Methylobacterium* genus among the non-methanotrophs, appear to possess a large number of predicted BGCs. The *Methylobacterium* genus was also highlighted for its substantial biosynthetic potential in a past analysis of bacterial genomes (Cimermancic *et al*., 2014). Many Alphaproteobacteria including methylotrophic species rely on the ethylmalonyl-CoA pathway to assimilate acetyl-CoA into central metabolism, and this pathway involves high flux through CoA-linked intermediates that are also often used in the synthesis of specialized metabolites such as polyketides (Alber, 2011). This makes the exploration of the specialized metabolism of these organisms particularly intriguing.

Bacteriocin and terpene BGCs were most commonly predicted within the categories classified by the antibiotic and secondary metabolite analysis shell (antiSMASH) (Blin *et al*., 2017). However, the majority of predicted clusters were detected using ClusterFinder, which relies on more probabilistic (and less stringent) methods for BGC identification (Cimermancic *et al*., 2014). This could suggest that methylotrophs produce different compounds than those made by traditionally studied organisms, which would be promising for the discovery of new classes of compounds. However, sequence homology-based comparisons to characterized BGCs and experimental verification will be essential to exploring the hypothesis that these gene clusters are producing novel products.

Intercellular chemical communication

Quorum sensing

Quorum sensing (QS) allows bacteria to control gene expression in a cell density-dependent manner. In Proteobacteria, a canonical QS signal is the acyl-homoserine lactone (acyl-HSL), which can vary in its acyl chain length, saturation, and oxidation. These signals are produced by LuxI-family acyl-HSL synthases, and detected by LuxR-family receptor/transcription factors. For reviews please see Papenfort and Bassler (2016) as well as Whiteley *et al*. (2017).

The first QS system to be characterized in a methylotroph was in the model strain *Methylobacterium extorquens* AM1 (Penalver *et al*., 2006). Researchers found that AM1 possesses two LuxI-family synthases, termed MlaI and MsaI. While MsaI produces the short chain acyl-HSLs C_6- and C_8-HSL, MlaI produces the more unusual unsaturated long

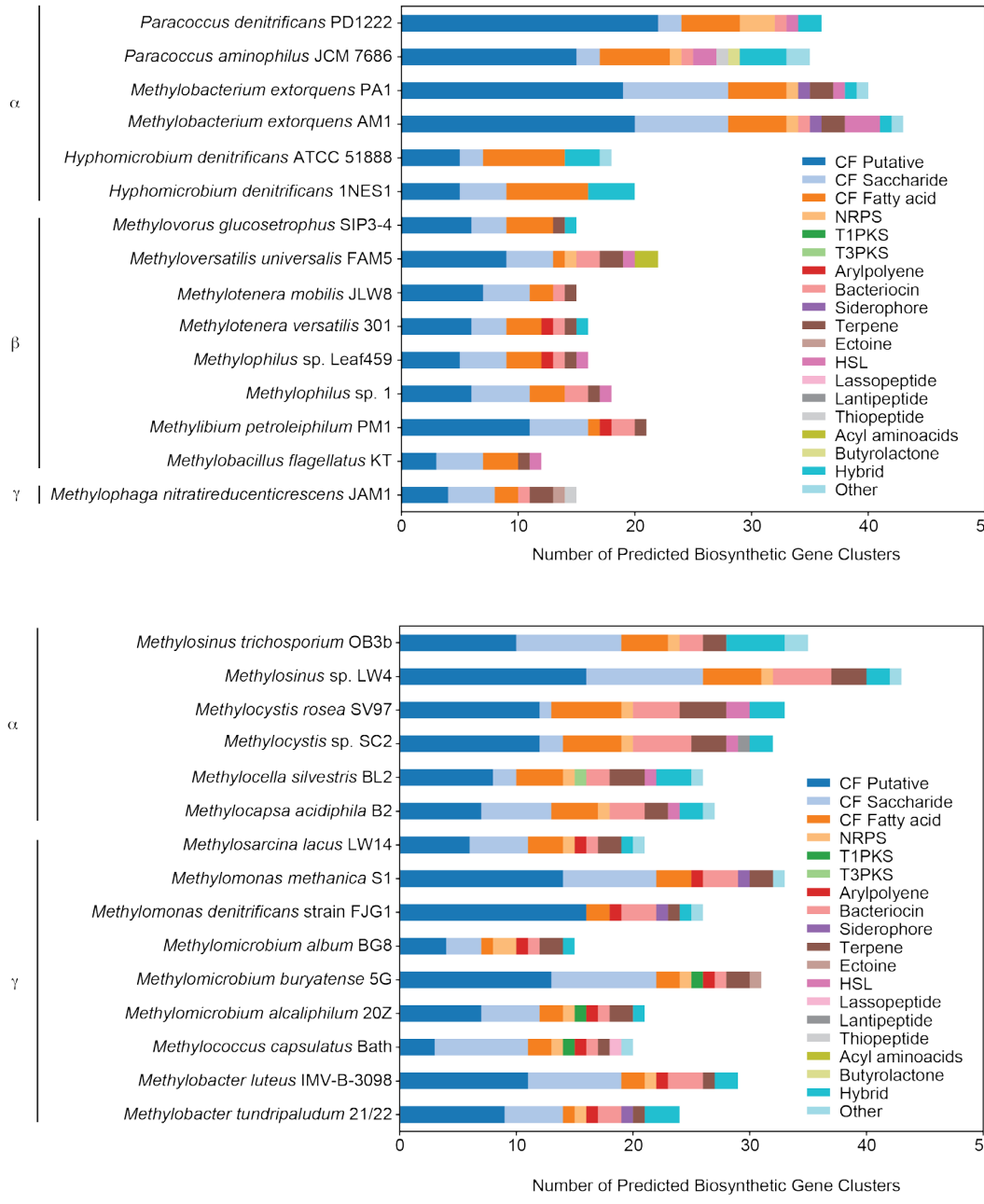


Figure 12.1 Predicted biosynthetic gene clusters in genomes of methylotrophic Proteobacteria. Predictions were made using antiSMASH 4.1.0 (Blin *et al*., 2017) with ClusterFinder (CF) (Cimermancic *et al*., 2014) enabled with default settings. HSL, homoserine lactone; NRPS, non-ribosomal peptide synthetase; PKS, polyketide synthase.

chain molecules 7*Z*-C_{14}-HSL and 2*E*,7*Z*-C_{14}-HSL only when AM1 is grown on methanol (Table 12.1). The short chain acyl-HSLs produced by *M. extorquens* AM1 increase the production of exopolysaccharide (Penalver *et al.*, 2006), and therefore could have a role in biofilm formation. In support of this, recently a highly homologous QS system in a *Methylobacterium populi* strain was found to regulate the structure and adherence of biofilms made by this organism (Morohoshi *et al.*, 2018).

Many *Methylobacterium* species have been found to produce acyl-HSLs as detected by bioassays (Poonguzhali *et al.*, 2007). In one study researchers found that the orange tree symbiont *Methylobacterium mesophilicum* SR 1.6/6 produces several signals, including novel variants (Table 12.1) (Pomini *et al.*, 2009). There is some evidence that increased concentrations of these molecules may regulate the transcription of metabolic genes in this bacterium (Dourado *et al.*, 2013), however the precise biological role of these QS signals will require further investigation.

A QS system was recently discovered and characterized in the methane-oxidizing Gammaproteobacterium *Methylobacter tundripaludum* 21/22, which was isolated from lake sediment (Table 12.1) (Puri *et al.*, 2017). This bacterium produces and responds to the signal 3-OH-C_{10}-HSL, which activates expression of a BGC co-located with the QS genes in *M. tundripaludum*. The same group of researchers subsequently identified the specialized metabolite product of this BGC, named tundrenone (see below) (Puri *et al.*, 2018). The fact that tundrenone is produced in a QS-dependent manner fits the paradigm that QS systems often activate the production of extracellular factors such as antibiotics (McGowan *et al.*, 1995; Duerkop *et al.*, 2009) and proteases (Pearson *et al.*, 1997), thereby allowing bacteria to affect their surrounding environment at high cell density.

It will be exciting to characterize more methylotroph QS systems in the future and determine their biological roles. It should be noted that methylotrophs occupy diverse ecological niches, including both acidic (Dedysh *et al.*, 2000) and alkaline (Khmelenina *et al.*, 1997) environments. The HSL ring is subject to base-catalysed hydrolysis (Schaefer *et al.*, 2000; Dong *et al.*, 2001), which may provide one explanation for why methylotrophs isolated from alkaline environments such as some *Methylomicrobium* species do not possess acyl-HSL-based quorum sensing systems in their genomes (Fig. 12.1).

Interspecies chemical communication

Specialized metabolites biosynthesized by methylotrophs have also been reported to be involved in interspecies interactions. The copper-binding small molecule methanobactin (see below) is produced by many methanotrophs, but its structure varies between species (Kenney and Rosenzweig, 2018a). It was reported that methanobactin produced by *Methylocystis* sp. str. SB2 may regulate the

Table 12.1 Acyl-homoserine lactone quorum sensing signals produced by methylotrophic Proteobacteria

Producing strain	Acyl-homoserine lactone signal	Reference
Methylobacterium extorquens AM1	C_6-HSL C_8-HSL 7Z-C_{14}-HSL 2E,7Z-C_{14}-HSL	Penalver *et al*. (2006)
Methylobacterium mesophilicum SR 1.6/6	C_{12}-HSL C_{13}-HSL C_{14}-HSL 2E-C_{12}-HSL 7Z-C_{14}-HSL 2E,7Z-C_{14}-HSL	Pomini *et al*. (2009)
Methylobacter tundripaludum 21/22	3-OH-C_{10}-HSL	Puri *et al*. (2017)
Methylobacterium populi P-1M	3-OH-C_{14}-HSL $C_{14:1}$-HSL[a]	Morohoshi *et al*. (2018)

Note: While the full stereochemistry has not been determined for all molecules, all biologically produced acyl-homoserine lactone (acyl-HSL) signals where stereochemistry has been determined possess an (*S*)-*N*- configuration.
[a]Stereochemistry of unsaturated bond not determined.

expression of genes related to methane oxidation in *Methylosinus trichosporium* OB3b via mechanisms beyond simply altering copper availability (Farhan Ul-Haque *et al.*, 2015). The ability of methanotrophs to interact with methanobactin produced by other species points to piracy between these organisms and suggests a fierce competition for copper in the environment (El Ghazouani *et al.*, 2012; Dassama *et al.*, 2016; DiSpirito *et al.*, 2016).

The presence of volatile specialized metabolites in interactions between methanotrophs and non-methylotrophic heterotrophs has also been surveyed (Veraart *et al.*, 2018). The methanotroph *Methylobacter luteus* was reported to produce the volatile bicyclic terpenoids cadinene and alpha-murolene in response to the presence of the heterotroph *Pseudomonas mandelli*. The role of these compounds in interactions between metabolically linked methanotrophs and non-methanotrophic species will require further investigation.

Examples of molecules produced by methylotrophs

Methanobactin

The particulate methane monooxygenase (pMMO) is found in almost all methane-oxidizing bacteria, and it requires copper to catalyse the oxidation of methane to methanol (Balasubramanian *et al.*, 2010). Consequently, methanotrophs have a great need for copper and use specialized mechanisms for its acquisition (Semrau *et al.*, 2010; Kenney and Rosenzweig, 2018b). Several methanotrophs that are members of the Alphaproteobacteria secrete a ribosomally produced and post-translationally modified peptide (RiPP) that tightly binds copper termed methanobactin (Table 12.2) (Kim *et al.*, 2004). Because of its relation to iron binding siderophores, methanobactin is known as a chalkophore. Methanobactin is perhaps the most well known of all characterized specialized metabolites produced by methylotrophs. For more comprehensive reviews of methanobactin please see DiSpirito *et al.* (2016) as well as Kenney and Rosenzweig (2018a).

Although the exact structure of methanobactin varies between species, all isolated versions coordinate copper using oxazolone rings or heterocycles with similar functionality and neighbouring enethiol/thioamide groups (Kenney and Rosenzweig, 2018a). The formation of the oxazole ring and thioamide in methanobactin was recently characterized, and involves the protein complex MbnBC, which contains domains of previously unrecognized function (Kenney *et al.*, 2018). Understanding the molecular mechanisms of methanobactin biosynthesis may ultimately enable the production of methanobactin analogues with varying properties.

Applications of methanobactin include its use as a chelation therapy for copper toxicity such as in Wilson disease (Lichtmannegger *et al.*, 2016). Methanobactin from *M. trichosporium* OB3b also binds other metals including gold with high affinity (Choi *et al.*, 2006), making it useful for biomining as well as nanoparticle preparations (DiSpirito *et al.*, 2016). This versatile compound also has growth inhibitory activity against a range of bacteria (DiSpirito *et al.*, 2007), which is a less discussed property of methanobactin but which may play a role in shaping the composition of methane-oxidizing bacterial communities in nature. Methanobactin production is activated by low copper availability, making it similar to other specialized metabolites that have tightly regulated production (Kim *et al.*, 2004; Kenney *et al.*, 2016; Gu and Semrau, 2017). Genome mining has also identified methanobactin BGCs in other non-methyltrophic species (Kenney and Rosenzweig, 2013), and it will be interesting to see what role methanobactins play in these bacteria.

Toblerols

Modular, non-iterative polyketide synthases (PKSs) follow an assembly line logic that often makes it possible to predict the structure of the metabolite product (Till and Race, 2016). The products of *trans*-acyltransferase (*trans*-AT) PKSs can be more difficult to predict, however detailed studies of the genes in these BGCs has resulted in a set of product prediction rules for these modular synthases as well (Nguyen *et al.*, 2008). Recently, researchers investigated an unusual *trans*-AT PKS gene cluster in *M. extorquens* AM1 that could not be classified using previously developed approaches (Ueoka *et al.*, 2017). The products of the cluster were found to be a family of novel molecules containing cyclopropanol moieties, and these compounds were subsequently named the toblerols (Table 12.2). The authors discovered that toblerol C suppresses the production of an additional factor

Table 12.2 Selected specialized metabolites produced by methylotrophic Proteobacteria

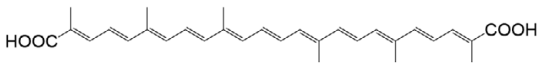
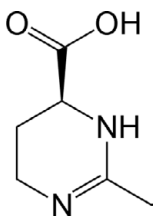
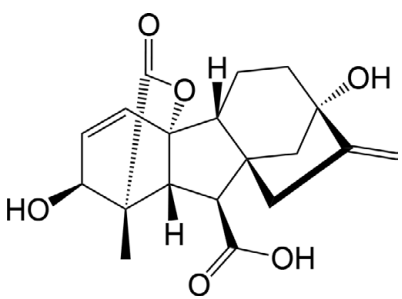
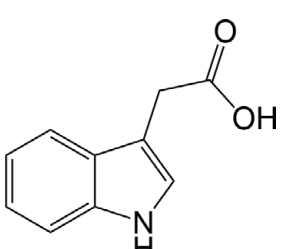
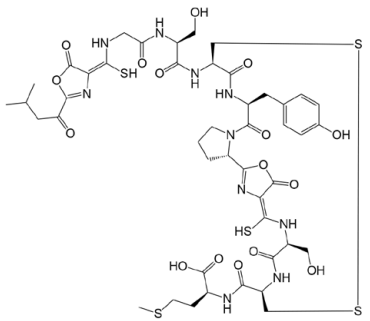
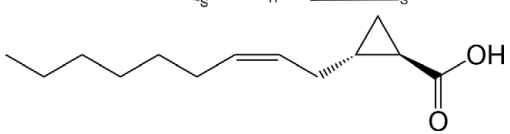
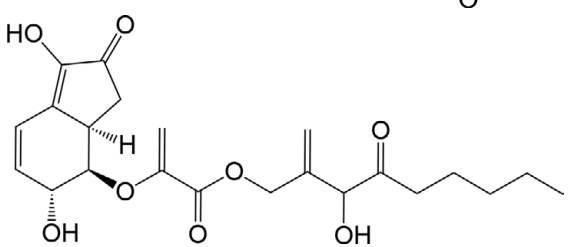
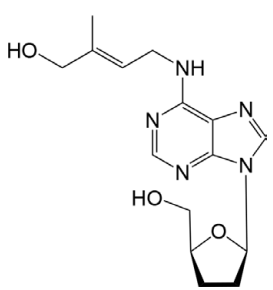
Compound name	Structure	Examples of producing strain(s)	Reference
4,4′-Diapolycopene-4,4′-dioic acid		*Methylobacterium rhodium*, *Methylomonas* sp. str. 16a	Kleinig *et al.*, (1979), Tao *et al.* (2005)
Ectoine		*Methylomicrobium alcaliphilum* 20Z	Reshetnikov *et al.* (2006)
Gibberellic acid (GA_3)		*Methylobacillus arboreus* Iva^T	Agafonova *et al.* (2018)
Indole-3-acetic acid (IAA)		*Methylobacterium mesophilicum* DSM 1708	Ivanova *et al*. (2001)

Table 12.2 Continued

Compound name	Structure	Examples of producing strain(s)	Reference
Methanobactin (from OB3b)		*Methylosinus trichosporium* OB3b (structural variants produced by other species)	Kim *et al.* (2004), Behling *et al.* (2008)
Toblerol C		*Methylobacterium extorquens* AM1	Uekoa *et al.* (2017)
Tundrenone		*Methylobacter tundripaludum* 21/22	Puri *et al.* (2018)
trans-Zeatin-riboside		*Methybacterium mesophilicum* DSM 1708, *Methylovorus mays* VKM B-2221, *Methylobacterium extorquens* DSM 1337	Ivanova *et al*. (2000), Koenig *et al*. (2002)

that inhibits the growth of other *Methylobacterium* isolates. The actual factor that causes growth inhibition is currently under investigation, but the results so far point to an intriguing hierarchy of bioactive small molecule production and regulation in these bacteria. This study highlights an example where a methylotroph contains a non-canonical BGC that produces novel specialized metabolites, demonstrating the utility of methylotrophs as an underexplored source of these molecules.

Tundrenone

Tundrenone is a novel specialized metabolite produced by the methanotroph *M. tundripaludum* in a quorum sensing-dependent manner (Puri *et al.*, 2017, 2018). Tundrenone contains a tetrahydroindenone core predicted to be derived from the primary metabolite chorismate, as well as a lipid tail with unusual modifications (Table 12.2). These notable structural features suggest that an understanding of tundrenone's biosynthesis may uncover new enzymatic reactions that are useful for understanding and engineering the biosynthesis of other small molecules. The biological function of tundrenone is currently unknown, however the BGC responsible for tundrenone production is conserved in *M. tundripaludum* isolates from across the globe (Puri *et al.*, 2017), suggesting that this molecule may have an ecologically significant role for this organism.

Carotenoids

Many methylotrophs are pigmented, as the responsible carotenoids can provide antioxidant effects including protecting their hosts from UV damage (Birben *et al.*, 2012; Vorholt, 2012). This fact also has led to the commonly used descriptive grouping of pink pigmented facultative methylotrophs (PPFMs), which includes many species of the *Methylobacterium* genus that are found in association with plants (see below). *Methylobacterium rhodium* (formerly *Pseudomonas rhodos*) produces C_{30}-carotenoids including 4,4′-diapolycopene-4,4′-dioic acid diesters that contain conjugated sugars (Table 12.2) (Kleinig *et al.*, 1979). These unusual molecules are not known to be produced by many bacterial species, but they were also found to be produced by methane-oxidizing bacteria of the genus *Methylomonas* (Tao *et al.*, 2005). An oxidase in strain 16a termed CrtNb has been identified and characterized that functionalizes the termini of the precursor molecule 4,4′-diapolycopene with aldehyde moieties (Tao *et al.*, 2005). This enzyme may be useful for future modification strategies for these pigments to be employed in semisynthetic processes.

Production of the more traditional C_{40} carotenoids has also been studied in methylotrophs. *Methylobacterium* species are known to produce C_{40} carotenoids such as oscillaxanthin (Van Dien *et al.*, 2003; Konovalova *et al.*, 2007). Additionally, *Methylomonas* sp. str. 16a has been used as a chassis for the production of the C_{40} carotenoid astaxanthin (Ye *et al.*, 2007). An astaxanthin-producing methanotroph could be used in aquaculture as a single cell protein source that simultaneously enhances the pigmentation of the farmed animals.

Ectoine

Ectoine is a cyclic imino acid that functions as an osmoprotectant in many species of halotolerant and halophilic bacteria, including several methylotrophic species found in ecosystems with high salinity (Table 12.2) (Galinski *et al.*, 1985; Reshetnikov *et al.*, 2011). The biosynthesis of ectoine has been studied in the halotolerant methane-oxidizing bacterium *Methylomicrobium alcaliphilum* 20Z (Reshetnikov *et al.*, 2005, 2006), which was isolated from a soda lake in Russia (Khmelenina *et al.*, 1997; Kalyuzhnaya *et al.*, 2008). Ectoine is produced from aspartic acid by the products of the *ectABC* biosynthetic gene cluster, and its production markedly increases under osmotic stress (Reshetnikov *et al.*, 2011). As with many specialized metabolites, the biosynthesis of ectoine is transcriptionally regulated. The *ectABC* BGC also contains a co-located MarR-type transcriptional repressor, which is partially responsible for regulation of *ectABC* expression at different salt concentrations (Mustakhimov *et al.*, 2010). Production of ectoine is of commercial interest because this compatible solute is valuable for the preservation of biologics including those used in cosmetics and medicine, and currently ectoine is produced biosynthetically for commercial means (Graf *et al.*, 2008; Kunte *et al.*, 2014). Consequently there is interest in production of this specialized metabolite from methane gas, a cheap and abundant feedstock for industrial biotechnology.

Phytohormones

Plant stomata release methanol during growth as a by-product of pectin demethylation (Nemecek-Marshall *et al.*, 1995; Galbally and Kirstine, 2002), and this methanol is in turn catabolized by resident methylotrophs that occupy the phyllosphere (Vorholt, 2012). In addition to this metabolic link, the relationship between plants and methylotrophs is thought to be mutualistic, with methylotrophs providing phytohormones to their plant hosts (Holland *et al.*, 2002; Kutschera, 2007; Fedorov *et al.*, 2011).

In support of the close link between plants and methylotrophs, *Methylobacterium* species have been found to promote the growth of plants, and the molecular details of these interactions have been investigated. For example, strains of *M. mesophilicum* and *Methylovorus mays* were found to produce substances that promote the growth of *Amarantus caudatus* seedlings, including the cytokinin zeatin (Ivanova *et al.*, 2000). *trans*-Zeatin biosynthesis in *Methylobacterium* spp. was later found to involve tRNA precursors (Koenig *et al.*, 2002); however, removal of *trans*-zeatin alone from bacterial cultures had no effect on soybean seed germination stimulation.

Methylotrophs have also been reported to synthesize additional phytohormones, including auxins and gibberellins. Researchers found that *M. mesophilicum* and *Aminobacter aminovorans* strains produce the canonical auxin indole-3-acetic acid (IAA) (Ivanova *et al.*, 2001), which exerts major influences on plant growth and development (Duca *et al.*, 2014). Furthermore, a *Methylobacterium oryzae* strain (Siddikee *et al.*, 2010) as well as the obligate methylotroph *Methylobacillus arboreus* (Agafonova *et al.*, 2018) have been reported to produce bioactive gibberellic acid GA_3, and GA_3 from the latter strain stimulated the sprouting of lettuce seeds.

These molecules point to a complex chemical conversation between methylotrophs and their plant hosts, as plants are canonical producers of natural products.

Conclusions

The specialized metabolites that have been isolated and characterized to date from methylotrophic Proteobacteria have led to the discovery of novel structures, molecular functions, and enzymatic transformations. These efforts also underscore the continued utility of isolating bacterial strains and characterizing the compounds they produce naturally. While culturing these organisms may involve specialized knowledge or equipment, doing so can aid in the discovery of a metabolite's biological activity due to biological context. Isolating naturally produced compounds can also add some certainty that a molecule of interest is the actual product of the native pathway, which can be an issue for discovery efforts based on heterologous expression. The predicted biosynthetic potential of methylotrophic Proteobacteria, along with several practical advantages based on the growth conditions of these bacteria, make this an exciting time to investigate the specialized metabolism of these organisms.

Future directions

It has become increasingly clear that many bacterial BGCs are not expressed in axenic cultures grown under laboratory conditions (Milshteyn *et al.*, 2014; Rutledge and Challis, 2015). Consequently, biological context is necessary for both the production of many specialized metabolites and an understanding of their bioactivity. Methylotrophic bacteria occupy a diversity of niches, and one particularly intriguing future direction for the study of specialized metabolites produced by these organisms is in the context of microbe-microbe and microbe–host interactions. Examples include plant–microbe interactions, where recently a *Methylophilus* strain was found to produce factor(s) that inhibit the growth of several other phyllosphere constituents (Helfrich *et al.*, 2018). Methylotrophs are also symbionts of marine animals (Cavanaugh *et al.*, 1987; Petersen and Dubilier, 2009), and a sponge symbiont with a large biosynthetic repertoire was recently predicted to have the ability to catabolize methanol (Lackner *et al.*, 2017). Finally, the metabolic relationships between methane-oxidizing bacteria and the non-methanotrophic heterotrophs they support in the environment may provide context for the discovery of new molecules (Oshkin *et al.*, 2014; Krause *et al.*, 2017; Veraart *et al.*, 2018). It will be exciting to see the developments in each of these areas in the years to come.

Web resources

- Antibiotics and secondary metabolite analysis shell (antiSMASH): https://antismash.secondarymetabolites.org/
- MIBiG Repository: https://mibig.secondarymetabolites.org/repository.html
- JGI Integrated Microbial Genomes and Microbiomes: https://img.jgi.doe.gov/
- Carotenoids Database: http://carotenoiddb.jp/

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