Methanotrophy: An Evolving Field

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As a field, methanotrophy has emerged in the early twentieth century, marked by the discovery of microbes that could sustain growth on methane gas, using it as the source of both carbon and energy. One hundred plus years later, the field is mature, having accumulated deep knowledge on different modes of methane metabolism, in microbes of different domains of life, bacteria and archaea, both aerobic and anaerobic. The past decade in methanotrophy has been marked by new important discoveries, including novel guilds of methanotrophs, novel metabolic modes, and novel enzymes and pathways, demonstrating that methanotrophy is an evolving field, and, likely, much is yet to be discovered. Future challenges include deciphering the mechanistic details of methane activation by the particulate methane monooxygenase, including the source of electrons in this reaction, understanding the respective functions of redundant enzymes such as alternative methane monooxygenases, methanol dehydrogenases, and other enzymes and pathways, and obtaining further insights into the evolution of methanotrophy, both aerobic and anaerobic. While methane is practically unlimited on this planet, thus presenting an attractive, renewable source of carbon for biotechnological use, including synthesis of fuels, multiple technical challenges exist in harnessing extant methanotrophs as efficient commercial platforms or, reversely, in engineering established platforms, such as *E. coli* or yeast, to utilize carbon from methane.

1.1 A Brief History of Methanotrophy

Methanotrophy is a field of study focused on metabolism of methane, carried out by microorganisms, and it is over 100 years old. Discovery of methanotrophy as a metabolic mode can be dated to circa 1906, when papers were published describing

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M. G. Kalyuzhnaya, X.-H. Xing (eds.), *Methane Biocatalysis: Paving the Way to Sustainability*, https://doi.org/10.1007/978-3-319-74866-5_1

microbes capable of growth on methane gas (Kaserer 1906; Söhngen 1906). The active exploration of the properties and the metabolic details of methanotrophy mostly date to the early 1970s, after Whittenbury and colleagues demonstrated that multiple cultures of methanotrophs can be isolated from a variety of environments, also describing media that support their growth in laboratory (Whittenbury et al. 1970). The protocol described by Whittenbury and the nitrate and ammonium minimal salts (NMS, AMS) media are still in use today (Dedysh and Dunfield 2017). Thin section microscopy of methanotrophic microbes revealed that their cells were peculiar in a way that they were filled with regular structures, recognized as internal membranes (Proctor et al. 1969), their presence appearing to be connected to methane-oxidizing activity (Anthony 1982). Moreover, two types of membrane structures were recognized, stacked, and peripherally distributed, suggesting that at least two different types of methanotrophs existed (Whittenbury et al. 1970). These types are now known, respectively, as gammaproteobacterial and alphaproteobacterial methanotrophs.

Insights into the biochemical pathways that enabled assimilation of methane carbon into biomass revealed that the differences between the two types of methanotrophs extended beyond membrane types and into central metabolism: the microbes possessing the stacked membranes assimilated carbon through condensation of formaldehyde with a sugar molecule, through the ribulose monophosphate (RuMP) cycle, and microbes with the peripheral membranes condensed formaldehyde with an amino acid, through the serine cycle. These different types are still referred to as Type I and Type II methanotrophs, respectively (Trotsenko and Murrell 2008). Early studies of the main enzyme in methanotrophy, the one that activates the highly inert molecule of methane, produced some controversial results, one group identifying a soluble multisubunit enzyme (Colby and Dalton 1978), another identifying a membrane-bound enzyme, whose subunits had different molecular masses (Tonge et al. 1977). The controversy was later solved by the realization that both forms exist, the soluble (sMMO) and the particulate (pMMO) methane monooxygenases, and these days, multiple structure solutions have been generated for both enzymes (Ross and Rosenzweig 2017). While the ultimate source of electrons for the sMMO is NADH, the source of electrons for pMMO or the exact mechanism of methane activation by this enzyme remain undefined (Ross and Rosenzweig 2017).

Downstream of methane, the enzyme responsible for oxidation of methanol, the pyrroloquinoline quinone (PQQ)-linked methanol dehydrogenase, has also been thoroughly analyzed (Anthony and Zatman 1964, 1965, 1967a, b), and this enzyme and the respective genes have been found highly conserved among Type I and Type II methanotrophs, as well as among non-methanotrophic methylotrophs (Lidstrom et al. 1994).

Enzymes/pathways for formaldehyde oxidation have also been analyzed, identifying multiple possible candidates, which included the dissimilatory RuMP cycle, the glutathione-linked formaldehyde oxidation pathway, the tetrahydrofolate (H_4F)-linked pathway, as well as putative NAD-linked and dye-linked formaldehyde dehydrogenases (Anthony 1982). As a rather surprising addition to all these potential

pathways for formaldehyde oxidation, a pathway has been uncovered in the late 1990s, involving reactions dependent on tetrahydromethanopterin (H_4MPT) and methanofuran (MF; Chistoserdova et al. 1998), previously characterized in anaerobic methanogenic archaea (Thauer 1998), and this pathway has been demonstrated to be widely distributed among different guilds of methylotrophs (Vorholt et al. 1999). The discovery of this pathway not only expanded the understanding of the metabolic potential of aerobic methylotrophs but also questioned the evolution of their metabolism, raising questions of how the same or very similar enzymes could carry out reactions key to "strictly aerobic" and "strictly anaerobic" metabolisms (Chistoserdova et al. 1998, 2004).

The details of the metabolic transformations constituting both the RuMP and the serine cycles were mainly deciphered by Quayle and colleagues, in series of studies simple in their elegance, mostly using labeling with radioactive carbon from methanol, formate or CO_2, followed by chromatographic analysis of the metabolites (Large et al. 1961, 1962a, b; Large and Quayle 1963; Kemp and Quayle 1965, 1966, 1967; Salem et al. 1972; Strøm et al. 1974). While modern approaches such as metabolomics and flux analysis can now be applied to precisely model carbon flux distribution among different reactions (Peyraud et al. 2009; Kalyuzhnaya et al. 2013; de la Torre et al. 2015), the pathways as outlined in the 1960s and 1970s remain true today, with perhaps one exception. The ethylmalonyl-CoA (EMC) pathway, a pathway for conversion of acetyl-CoA, functioning as part of the serine cycle, has remained a mystery for about 50 years since the deficiency of some of the serine cycle methylotrophs in the glyoxylate cycle has been discovered (Anthony 2011), being completely resolved only between 2007 and 2009 (Erb et al. 2007, 2009). While it remains unknown why some methylotrophs use the glyoxylate cycle, some use the EMC pathway, and some use both for either methylotrophy or acetate metabolism (Chistoserdova 2011); a similar situation exists in archaea, some of which utilize the glyoxylate cycle and some utilize an alternative methylaspartate cycle, which, in turn, shares some of the reactions with the EMC pathway (Khomyakova et al. 2011).

The process of anaerobic oxidation of methane (AOM) has also been known for a long time, based on the geochemical evidence (Reeburgh 1976, 1980). The microbes involved in this process were identified relatively recently, and these were found to be archaea and not bacteria (known as ANME-type archaea) (Hinrichs et al. 1999; Boetius et al. 2000; Orphan et al. 2001; Knittel and Boetius 2009). The early metagenomics studies suggested that methanotrophy must be carried out by these species using a reverse methanogenesis pathway (Hallam et al. 2004), which, with the exception of the early reactions transforming methane into a methyl moiety attached to coenzyme M (Scheller et al. 2010), would be similar to the oxidation of formaldehyde carried out through H_4MT and MF-linked reactions by the bacterial methanotrophs (Chistoserdova et al. 2004). Methane carbon was proposed to be assimilated via the Wood-Ljungdahl pathway (Hallam et al. 2004) that is also used by both the methanogenic archaea and the anaerobic methylotrophic clostridia (Drake et al. 2008). Thus, while the aerobic and the anaerobic modes of methane oxidation were considered fundamentally different, they both involved several

common reactions and cofactors, again questioning the commonality of their evolution (Braakman and Smith 2012; Weiss et al. 2016).

1.2 The Past Decade in the Methanotrophy Field

While progress in methanotrophy research has been steady over the past 50 years or so, it seems to have especially accelerated over the past decade (Fig. 1). This acceleration may be partly attributed to the wide application of modern tools such as genomics and other systems approaches (Chistoserdova 2017) and partly perhaps to the renewed interest toward methanotrophs as prospective targets for biotechnological platform development (Kalyuzhnaya et al. 2015; Strong et al. 2015). The past 10 or so years saw discovery of novel guilds of methanotrophs, as well as of novel enzymes and pathways, along with several corrections to the metabolic modes characterized in the past. A brief review of these recent and exciting discoveries is presented below.

Two new guilds of methanotrophs have been recently discovered within the bacterial domain of life, phylogenetically distinct from the proteobacterial methanotrophs and belonging to *Verrucomicrobia* (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008; Op den Camp et al. 2009) and to the candidate phylum NC10 (Raghoebarsing et al. 2006; Ettwig et al. 2010). These discoveries likely suggest that methanotrophy may be occurring within the bacterial domain of life even more widely and new methanotroph species are awaiting to be discovered. Despite the long phylogenetic distances between *Proteobacteria*, *Verrucomicrobia*, and NC10 bacteria, the metabolic scheme for methane oxidation in the newly discovered guilds is similar to the one in *Proteobacteria*, and it proceeds all the way to CO_2, which is then assimilated via the classical Calvin-Benson-Bassham cycle (Khadem et al. 2011; Rasigraf et al. 2014), presenting a novel combination of the assimilatory/dissimilatory modules enabling methanotrophy (Chistoserdova 2011).

A novel methanol dehydrogenase (MDH) has also been discovered. While a gene, named *xoxF*, along with the respective protein XoxF, has puzzled the methylotrophy community for a long time (Chistoserdova 2011), and while evidence was available for this enzyme to have a function in methylotrophy (Mustakhimov et al. 2013), low activity with methanol (Schmidt et al. 2010) continued to suggest that something was amiss. The missing factor turned out to be rare Earth elements (REEs), playing a catalytic role in XoxF enzymes (Hibi et al. 2011; Fitriyanto et al. 2011; Nakagawa et al. 2012), instead of calcium, the cofactor for the classic, MxaFI MDH enzyme (Anthony 2004). The hint on the catalytic role of REEs came from outside of the methylotrophy field (Hibi et al. 2011; Fitriyanto et al. 2011; Nakagawa et al. 2012), and their potential significance has not been embraced right away. However, in the recent few years, the research in REE-dependent methanol oxidation has been exploding, demonstrating, in a variety of key model organisms, that not only REEs are involved in methanol catalysis but that they are also involved in inverse regulation of genes for alternative MDH enzymes (Pol et al. 2014; Vu et al.

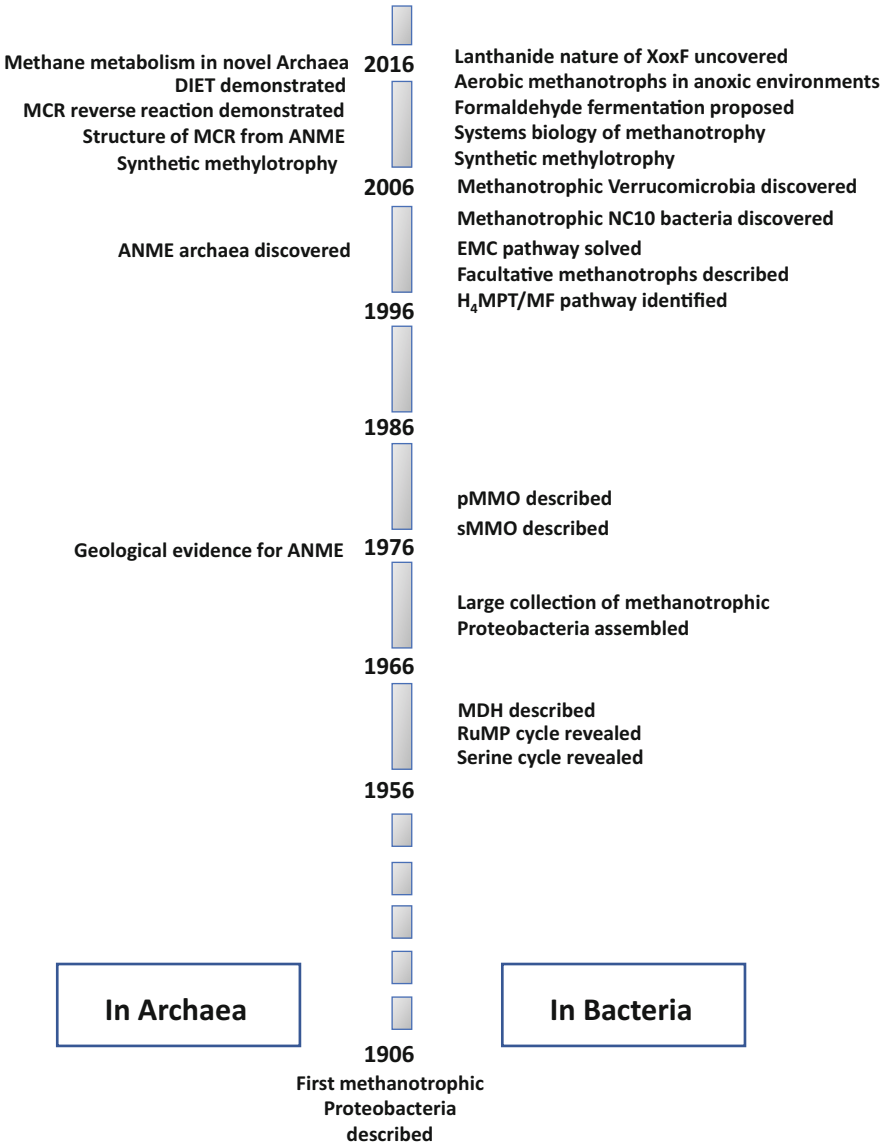

Fig. 1.1 Schematic of timeline for landmark discoveries in methanotrophy

2016; Chu and Lidstrom 2016, Chu et al. 2016; Gu et al. 2016). Moreover, XoxF-type MDH appears to be more environmentally widespread and more divergent than MxaFI enzymes, suggesting its ancestral origin (Chistoserdova 2011, 2015, 2017; Keltjens et al. 2014). The verrucomicrobial methanotrophs so far appear to only encode XoxF (Pol et al. 2014), the NC10 methanotrophs encode both MxaFI and XoxF (Ettwig et al. 2010), and proteobacterial methanotrophs encode either both

enzymes or only XoxF (Chistoserdova 2011; Vekeman et al. 2016; Padilla et al. 2017).

The major adjustments to the known methanotrophy pathways included the EMC pathway, as mentioned above, which changed the accepted balance between carbon from methane versus CO_2 carbon assimilated by these microbes (Anthony 1982), from 2:1 to 1:1 (Peyraud et al. 2009; Chistoserdova et al. 2009), highlighting the potential for these microbes in sequestering CO_2. Ironically, exactly this ratio was experimentally measured by the Quayle group in the 1960s (Large et al. 1961). The understanding of the metabolism of the RuMP cycle methanotrophs has also been adjusted to the original proposal by the Quayle group (Strøm et al. 1974), by uncovering that the glycolysis pathway is part of the RuMP cycle, along with the Entner-Doudoroff pathway (Kalyuzhnaya et al. 2013). Moreover, a formaldehyde fermentation pathway has been proposed utilizing reactions of the glycolysis pathway, as a metabolic mode for conditions of limited oxygen (Kalyuzhnaya et al. 2013).

Of the other dogmas established in the past century, the dogma of "obligate" methanotrophy, first questioned in 2005 (Dedysh et al. 2005), has been further dismantled, as least for the alphaproteobacterial methanotrophs (Semrau et al. 2011; Crombie and Murrell 2014; Dunfield and Dedysh 2014). In the gammaproteobacterial methanotrophs, the operation of the complete citric acid cycle, in the classic oxidative direction, has also been demonstrated (Fu et al. 2017). One of the most intriguing recent observations on "aerobic" methanotrophs that deviates from the doctrine is the apparent propensity of "aerobic" methanotrophs, especially representatives of the genus *Methylobacter*, to thrive in anoxic environments (Martineau et al. 2010; Graef et al. 2011; Tveit et al. 2013, 2014; Blees et al. 2014; Crevecoeur et al. 2015; Osvald et al. 2015, 2016a,b; Padilla et al. 2017; Martinez-Cruz et al. 2017). A denitrification capability has been uncovered in both proteobacterial methanotrophs and methanotrophs of the NC10 phylum, suggesting alternative electron acceptors (Ettwig et al. 2010; Kits et al. 2015). In the case of NC10 bacteria, a novel mechanism for intracellular O_2 production has also been proposed (Ettwig et al. 2010). However, activity of "aerobic" methanotrophs has been demonstrated in environments devoid of nitrate/nitrite (Milucka et al. 2015), suggesting alternative metabolic scenarios. It has been proposed recently that cryptic oxygen cycling is common in seemingly anoxic environments due to tight coupling of oxygen production and consumption, thus keeping oxygen at levels as low as subnanomolar (Garcia-Robledo et al. 2017).

Significant progress has been also made in understanding methane oxidation by the archaea. Reverse reaction activity for methyl-CoM reductase (MCR) has been experimentally demonstrated, supporting the role of this enzyme in primary methane oxidation by archaea (Scheller et al. 2010). In further support, the MCR homolog from a microbial mat active in methane oxidation revealed striking structural similarities with MCR enzymes involved in methanogenesis (Shima et al. 2011), providing firm evidence that methane production and methane oxidation must rely on the same enzyme. Moreover, it has even been demonstrated that ANME-type archaea can both produce and oxidize methane; this conclusion based on

quantification of gene transcripts of ANME in zones of methane oxidation and methane production, separated across the depths of a sediment (Lloyd et al. 2011).

Further progress has also been made toward resolving the potential mechanisms for interspecies electron transfer that is essential for anaerobic methane oxidation (Boetius et al. 2000; Orphan et al. 2001). The latest proposals favor direct electron transfer (DIET) between ANME and sulfate-reducing bacteria, which is mediated by pili as well as by multiheme cytochromes (McGlynn et al. 2015; Wegener et al. 2015; Krukenberg et al. 2016). Further support for DIET was obtained through decoupling AOM from sulfate reduction using artificial electron acceptors (Scheller et al. 2016). Methane oxidation by ANME linked to denitrification has also been discovered (Haroon et al. 2013; Arshad et al. 2015), this metabolism also involving a syntrophic partner, the anaerobic ammonia-oxidizing bacteria (Haroon et al. 2013). Moreover, novel lineages of archaea have been recently identified through culture-independent experiments with a potential in methane metabolism, belonging to novel phyla, Thorarchaeota (Seitz et al. 2016), Bathyarchaeota (Evans et al. 2015; Mwirichia et al. 2016; Lazar et al. 2016), and candidate phylum Verstraetearchaeota (Vanwonterghem et al. 2016). It remains to be demonstrated whether these novel organisms are active in methane oxidation, methanogenesis, or both.

Overall, discovery of novel phyla within both bacteria and archaea capable of methane transformations, including species possessing H_4MPT/MF functions not yet assigned to any specific metabolic pathway, further suggests the common evolutionary history for methanotrophy and methanogenesis (Chistoserdova 2013, 2016) and the ancient nature of these reactions (Weiss et al. 2016).

Another concept in methanotrophy that received recent support is the communal nature of the microbial metabolism of methane. The syntrophic nature of anaerobic methane oxidation by the archaea has been recognized from the very start, supported by bioenergetic constraints of this process (Thauer and Shima 2008). The anaerobic NC10 bacteria appear to also be syntrophic, as they still have not been cultivated in pure form. However, while the proteobacterial methanotrophs can be cultivated in pure cultures, they as well tend to form consortia with other, non-methanotrophic organisms (Dedysh and Dunfield 2017). Moreover, recent experiments questioning the composition of such consortia have identified co-occurrence patterns suggesting some type of specificity in methanotroph/non-methanotroph associations (Hernandez et al. 2015; Oshkin et al. 2015). While some potential metabolic linkages have been identified such as sharing of methanol (Krause et al. 2017; Tavormina et al. 2017), whether these are guild-level or species-/strain-level linkages remains to be determined.

1.3 Future Challenges

The field of methanotrophy has come of age, having accumulated sophisticated knowledge on the details of both oxygen-dependent and oxygen-independent microbial processes converting methane into energy and biomass. Yet the past decade in methanotrophy has been marked by series of new and exciting discoveries that

identify novel directions in methanotrophy and also pose novel challenges to be addressed in the future. Some of these are of academic value, and some are relevant to the potential industrial applications of methanotrophs. In terms of the former, the details of the activation of methane by the pMMO remain to be uncovered, along with the source(s) of electrons for the activation. This problem has become even more profound in the light of the recent evidence of the activity of "aerobic" methanotrophs in anoxic environments. Thus, along with the catalytic mechanisms, questions need to be resolved of how oxygen molecules are being sensed, accessed, stored, and transferred to the pMMO. Are the electrons involved in enzyme activation coming from within or from outside the cell, and can pMMO switch between different sources of electrons? Are there similarities between "aerobic" and "anaerobic" methanotrophs with this respect? Interestingly, multiheme cytochromes, akin to the cytochromes characterized in electricity-generating bacteria (Lovley 2017) or in ANME-type methane oxidizers (McGlynn et al. 2015; Wegener et al. 2015; Krukenberg et al. 2016), have been identified in some aerobic methanotrophs (Karlsen et al. 2011). Another question that still remains is whether sMMO and pMMO are redundant or whether they are tailored to specific metabolic goals. Likewise, the history and the distinct functions of the alternative methanol dehydrogenases (XoxF vs. MxaFI) in methanotrophy need to be further addressed, as well as the role of REEs in methane oxidation. So far, the published research on lanthanides in methylotrophy has used unnaturally high concentrations of REEs (Hibi et al. 2011; Fitriyanto et al. 2011; Nakagawa et al. 2012; Pol et al. 2014; Vu et al. 2016; Chu and Lidstrom 2016, Chu et al. 2016; Gu et al. 2016), which in turn, resulted in quick selection of mutants with modified behavior with relation to REEs (Chu et al. 2016). However, natural concentrations of REEs belong in a dramatically different range (Amyot et al. 2017; Turetta et al. 2017), posing questions whether, instead of the so-called lanthanide switch (Chu et al. 2016), a fine-tuned synergy exists between XoxF-type and MxaFI-type MDH enzymes and whether methanotroph communities compete for or share REEs. The questions about the evolution of methanotrophy are also becoming more intriguing as the recent data present more possibilities, given the facts that the "aerobic" methanotrophs are not so aerobic after all (Danilova et al. 2016), that common pathways are widespread among different guilds of "aerobic" and "anaerobic" methanotrophs, and that autotrophy now appears rather common in methanotrophy. The role of syntrophies in "aerobic" methane oxidation needs to be further questioned, in experiments with natural as well as synthetic communities, which may present novel models for studying methanotrophy (Yu and Chistoserdova 2017).

Nowadays, the significance of methane as a carbon source that could be utilized by the modern humanity has been increasing, considering that methane is practically unlimited on this planet, and that removal of methane, steadily produced by both natural and anthropogenic sources, and its conversion into value-added compounds, including fuels, would present the most practical solution to both greenhouse effect mitigation and to harvesting an abundant and sustainable carbon compound. While methanotrophs present attractive biotechnological platforms, at least theoretically (Kalyuzhnaya et al. 2015), many challenges exist that prevented their broad use on

large and commercially feasible scales (Strong et al. 2015). A reverse approach, of engineering some of the well-developed and commercially feasible platforms, such as *E. coli* or yeast, to consume methane with an output of value-added compounds, has also been challenging, especially in terms of integration of the methane oxidation module(s), and so far, such an approach lacks any evidence of a positive outcome. However, success was reported with engineering *E. coli* capable of converting methanol into value-added compounds (Whitaker et al. 2017). Success was also reported with engineering a methane-consuming recombinant archaeon, expressing archaeal methane oxidation module (Soo et al. 2016). Thus, both approaches, of engineering native methane oxidizers as well as recombinant strains, are worth pursuing in the future, as the new tools and technologies keep pushing the technical limitations and as, at the same time, the range of the organisms of potential commercial interest, including communities versus single cultures, is constantly broadening. Whether the methanotrophs (or methanotrophy) are ever fully harnessed in commercial applications, they will never cease to be an exciting group of organisms, possessing a unique capability of converting methane into biomass, aerobically or anaerobically.

Acknowledgments Support by the US Department of Energy (DE-SC-0016224) is acknowledged.

Conflict of Interest The author declares no conflicts of interest.

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