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Citation for published version:
Dexter, J, McCormick, AJ, Fu, P & Dziga, D 2021, 'Microcystinase – a review of the natural occurrence, heterologous expression, and biotechnological application of MlrA', *Water Research*, vol. 189, 116646. https://doi.org/10.1016/j.watres.2020.116646

Digital Object Identifier (DOI):
10.1016/j.watres.2020.116646

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Water Research

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Title

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Abstract

Microcystinase (MlrA) was first described in 1996. Since then MlrA peptidase activity has proven to be both the most efficient enzymatic process and the most specific catalyst of all known microcystins detoxification pathways. Furthermore, MlrA and the MlrABC degradation pathway are presently the only enzymatic processes with clear genetic and biochemical descriptions available for microcystins degradation, greatly facilitating modern applied genetics for any relevant technological development. Recently, there has been increasing interest in the potential of sustainable, biologically inspired alternatives to current industrial practice, with note that biological microcystins degradation is the primary detoxification process found in nature. While previous reviews have broadly discussed microbial biodegradation processes, here we present a review focused specifically on MlrA. Following a general overview, we briefly highlight the initial discovery and present understanding of the MlrABC degradation pathway, before discussing the genetic and biochemical aspects of MlrA. We then review the potential biotechnology applications of MlrA in the context of available literature with emphasis on the optimization of MlrA for *in situ* applications including (i) direct modulation of Mlr activity within naturally existing populations, (ii) bioaugmentation of systems with introduced biodegradative capacity via whole cell biocatalysts, and (iii) bioremediation via direct MlrA application.

Keywords

Microcystins; MlrA; Harmful Cyanobacteria; Bioremediation; Harmful Algae

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Abbreviations

AA	Amino Acid Residues
Adda	((2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid)
DOC	Dissolved Organic Carbon
EDTA	Ethylenediaminetetraacetic acid
GMM	Genetically Modified Microorganism
GST	Glutathione S-Transferase
HAB(s)	Harmful Algal Bloom(s)
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
MBP	Maltose Binding Protein
MCs	Microcystins
MS	Mass Spectrometry
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction

1. Introduction

Several recent reports have highlighted the increasing global occurrence, intensity, and duration of harmful algal blooms (HABs) (Ho et al., 2019; Svirčev et al., 2019; Tokodi et al., 2019). These trends are projected to continue and are attributed to expanding commercial agricultural practices and the impact of climate change. Microcystins (MCs) are the most widely distributed and abundant toxins associated with freshwater HABs (Bouaïcha et al., 2019). MCs are monocyclic heptapeptides comprised of both proteinogenic and non-proteinogenic amino acids, which are synthesized via nonribosomal peptide synthases in several genera of cyanobacteria. There are more than 250 unique chemical structures identified as MCs, although there is great disparity in both total environmental presence and in toxic effect (Chernoff et al., 2020). Specific MCs are designated via a suffix denoting the 2^{nd} and 4^{th} position amino acids within the heptapeptide, as these frequently vary. For example, microcystin-LR (MC-LR), one of the most toxic and commonly observed MCs, contains Leu (2^{nd} position) and Arg (4^{th} position) (see Bouaïcha et al. (2019) for further details).

In general, MCs are potent hepatotoxins/carcinogens that inhibit the activities of protein phosphatases 1 and 2A (Yoshizawa et al., 1990). There is also evidence that chronic, sub-acute exposure to MCs results in accumulation in liver tissue (Greer et al., 2018), and that bioaccumulation in both agriculture (Lee et al., 2017; Xiang et al., 2019) and aquaculture (Hu et al., 2018) may be significant routes of human exposure. Furthermore, the cyclic structure of MCs significantly enhances their recalcitrance to conventional water utility physicochemical treatments. In 1998, the World Health Organization established a provisional guideline value of 1 μg l^{-1} for the highest acceptable level of total MCs-equivalents in drinking water. Conventional

81 water treatment can be effective at removing low levels of waterborne MCs, yet significant
82 oxidative input is required after filtration of live MCs producers (i.e. to remove intracellular MCs
83 load) and cyanobacterial bloom conditions can often overwhelm water treatment capacity. In
84 addition, while advanced oxidation and adsorption-based processes (e.g. employing ozone and
85 activated carbon, respectively) are more successful at MCs removal, the efficacy of such
86 techniques is significantly affected by water quality parameters, such as Dissolved Organic
87 Carbon (DOC) and pH. More importantly, the costs associated with scaling these advanced
88 technologies to utility-scale often render their application as impractical and uneconomical,
89 especially considering application in economically developing regions (Sharma et al., 2012;
90 Charlebois, 2016; Ampiaw et al., 2019).

91 There is now increasing interest in developing more sustainable biologically based
92 methods to detoxify MCs contaminated waters, as biological degradation of MCs is the primary
93 natural process for remediation. Several genera of bacteria displaying degradation activity
94 against MCs have been identified from environmental samples. In terms of previous reviews,
95 Dziga et al. (2013a) gave a detailed account of microbial MCs biodegradation integrating the
96 contemporary knowledge of MlrA, while Kormas and Lymperopoulou (2013) presented a
97 complementary meta-analysis of the available 16S rRNA and *mlrA* gene diversity. Li et al. (2017)
98 presented a detailed discussion on the potential applications of MCs biodegradation and future
99 research directions. Massey et al. (2018) then provided a timely review of applied bacterial-
100 based biodegradation of MCs, which included a detailed listing of individual bacterial species
101 and mixed consortia that have been shown to degrade MCs, the conditions tested, and the MCs
102 degradation rates and degradation products. Massey and Yang (2020) provided the most recent

of all reviews of microbial MCs degradation referenced here, with additional emphasis on the toxic effects of MCs. Thus, the published work on microbial MCs biodegradation has been well considered. Nevertheless, no reviews to date have focused specifically on MlrA of the Mlr-dependent degradation pathway. MlrA is the key first enzyme involved in this process, with recent reports supporting the potential for environmentally conscious industrial application. Below provides brief overview of the ecological context of the Mlr-dependent pathway.

Despite the existence of multiple MCs degradative pathways, most of which have not been fully characterized, it is the ecological presence of the *mlr* gene cluster that has been directly associated with greatly accelerated MCs degradation, compared to bacterial consortia utilizing *mlr*-independent degradation pathways (Lezcano et al., 2016; Morón-López et al., 2017). The *mlr* gene cluster was originally isolated and characterized from the aerobic, gram-negative bacterium strain *Sphingomonas* sp. ACM-3962, formerly MJ-PV (hereafter ACM-3962) (Jones et al. 1994; Bourne et al., 1996). Subsequent ecological detections have also generally occurred under aerobic conditions, although *mlr* is not universally present nor required for MCs biodegradation within aerobic environments (hence both *mlr*-independent MCs biodegradation pathways and anaerobic MCs biodegradation pathways). Table 1 lists the verified *mlr* genes for bacterial strains shown to possess an *mlrA* homolog, with section 2 describing experimentally verified gene functions.

For further consideration of reports where *mlr*-dependent pathways were observed as undetectable/underrepresented compared to *mlr*-independent degradation pathways, we recommend Manage et al. (2009), and Kansole and Lin (2016). Also, investigations surrounding Lake Erie of the United States Great Lakes network should be considered (Mou et al., 2013;

Krishnan et al., 2018; Krausfeldt et al., 2019), with the technological potential of *mlr*-independent MCs degrading organisms initiated by Thees et al. (2018). Very recent metagenomic analyses from Jankowiak and Gobler (2020) demonstrated that microbiomes associated with surface attachment to *Microcystis* colonies have an increased abundance of *mlr* genes compared to the surrounding free-living microbial community structure. This observation contrasted previous studies by (i) Mou et al. (2013), which reported metagenomic *mlr* underrepresentation compared to *mlr*-independent pathways (xenobiotic metabolism genes, such as Glutathione S-Transferase [GST]) and (ii) Thees et al. (2018), which reported the absence of *mlr* within MCs degrading bacteria isolated from Lake Erie HABs. This disparity was thus attributed to sampling of the free-living microbial community in these previous reports (Jankowiak and Gobler, 2020). Interestingly, *Sphingosinicella microcystinivorans* strain Y2 (hereafter Y2) was isolated from within the extracellular mucilage of *Microcystis*, with a high correlation between Y2 population and the concentration of mucilage-bound MCs (Maruyama et al., 2003).

Originally, MlrA (microcystinase) was thought to be a metalloprotease, although recent structural modeling of both protease homology and substrate binding suggests that it is actually a glutamate intramembrane protease member of the type II CAAX prenyl endopeptidases (Xu et al., 2019b). This hypothesis still requires experimental verification. Well confirmed research has shown that MlrA acts specifically at the peptide bond between the 5^{th} position amino acid ((2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid) commonly referred to as Adda, which is a non-proteinogenic amino acid found in toxic cyanobacterial peptides like MCs, and the variable 4^{th} position amino acid. This catalyzing the

147 initiating reaction in the *mlr*-dependent degradation pathway (Bourne et al., 1996), which
148 linearizes the heptapeptide ring structure in MCs and results in a 2100-fold decrease in toxicity,
149 essentially rendering a non-toxic product (Dziga et al., 2012). MlrA is active against MCs
150 demonstrating a range of hydrophobicity due to commonly observed amino acid substitutions
151 at the 2nd and 4th positions, for example MC-LR, -RR, -YR, -LY, -LF and -LW (Dziga et al., 2012).
152 However, the specificity of MlrA towards these substrates may vary, with lower degradation
153 rate of MC-LW, -LF (Dziga et al., unpublished) (see section 3.2 for further discussion). In
154 conclusion, Figure 1 provides an overview of the major directions of previous and current
155 research on *mlrA*-dependent MCs biodegradation.

156

157 **2. Initial characterization and present understanding of the MlrA-mediated MCs degradation**
158 **pathway**

159 The groundbreaking first description of MlrA activity in ACM-3962 by Bourne et al.
160 (1996) offered the initial interpretation of biochemical evidence for the existence of a multi-
161 step enzymatic pathway responsible for degradation of MC-LR. Degradative activity was
162 observed toward MC-LR and MC-RR, but not nodularin. Nodularin is a monocyclic non-
163 ribosomal pentapeptide structurally similar to MCs, with an Arg-Adda peptide bond that is the
164 specific site of MlrA peptidase activity. Intriguingly, MlrA homologs described later have
165 demonstrated nodularin degradation (Imanishi et al., 2005; Xu et al., 2019a; Wu et al., 2019a).
166 This may suggest variance in the specificity of MlrA homologs and/or may be indicative of assay
167 protocols not optimized for the slower nodularin degradation rates later observed (Xu et al.,
168 2020). Returning to the work of Bourne et al. (1996), application of EDTA to ACM-3962 cellular

extracts during an MC-LR degradation assay resulted in varying inhibition of the Mlr enzymes, with HPLC analysis presenting both (i) a transient (intermediate) peak, and (ii) a stable, secondary peak. The identity of these peaks was confirmed by ion spray mass spectrometry (MS), with product (i) a linearized (acyclo-) MC-LR (N-terminal Adda), and product (ii) a tetrapeptide (NH_2-Adda-Glu(iso)-Mdha-Ala-OH). Product (ii) could also undergo further enzymatic degradation to smaller peptides/amino acids, but these were not structurally determined. MS data for product (i) clarified the MCs site of MlrA peptidase activity. The enzyme inferred responsible for product (i) generation (later termed MlrA) was suggested to be a metalloprotease, based on screening of protease inhibitors. The enzyme responsible for producing the product (ii) tetrapeptide (later termed MlrB) was suggested to be a bacterial serine protease (family II). Description of linearized molecules of both MC-LR and nodularin had been previously reported, but this earlier report suggested that these linear forms were precursors in the respective biosynthesis pathways (Choi et al., 1993). Despite this, Choi et al. (1993) noted that the linearized molecules lacked the toxicity of the cyclic structures via a mouse bioassay. This finding was further supported by a later mouse bioassay performed by Bourne et al. (1996), with an additional *in vitro* protein phosphatase activity assay noting the linearized MC-LR having 160-fold lower inhibitory activity (toxicity) than cyclic MC-LR.

Bourne et al., (2001) continued the experimental trajectory with the following accomplishments: (a) cloning and characterization of the complete *mlrABCD* genetic fragment, including first dissemination of complete, specified gene sequences, (b) the first description of heterologous expression of MlrA (336 AA, M_r 36.3 kDa; see Table 2 for a summary of all heterologous expression systems), and (c) the first discussion of the $H^{260}AIH^{263}NE^{265}$ motif

(HAIHNE) present within ACM-3962 MlrA, which is noted as a potential variant of the classical metalloprotease zinc-binding motif (HEXXH). The HAIHNE motif is conserved in all MlrA so far described. In addition, SignalP software analysis (Nielsen et al., 1997) predicted the presence of an N-terminal signal peptide targeting MlrA to the periplasmic space, with cleavage site between Ala_{26} and Leu_{27}. These two publications by Bourne et al. established the specific protease activity of MlrA on MC-LR, along with providing a first complete gene sequence, with indications of successful heterologous expression and, in practical terms, complete detoxification of MC-LR.

The current status of the total *mlr*-dependent degradation pathway, which is fundamentally dependent on initial linearization via MlrA, is informed by the following reports and presented in Figure 2 with MC-LR as model substrate. Hashimoto et al. (2009) further detailed the degradation of linear MC-LR and tetrapeptide and reported the observance of 3 tripeptides, 1 dipeptide, and 3 amino acids (Adda, Arg, and methylamine) via *Sphingomonas* strain B-9 (later given as *Sphingosinicella microcystinivorans* strain B-9, hereafter B-9). Ding et al. (2018) continued this effort with *Sphingopyxis* sp. m6. The latter study observed 3 tripeptides (identical to Hashimoto et al., 2009), 3 dipeptides, and 3 amino acids (Adda, Arg, Leu), and as such a revised degradation scheme was proposed. Via heterologous expression, Dziga et al. (2016) provided further clarification of the proteolytic action of MlrB and MlrC, with MlrC noted to specifically release Adda via hydrolysis of the peptide bond between Adda and Glu, and with MlrC catalytic activity towards both linear heptapeptide and tetrapeptide. MlrB showed peptidase activity between the position 1 and position 2 AA of both the linear heptapeptide and hexapeptide that is resultant from initial sequential MlrA and MlrC activity.

213 Most interestingly, first evidence is provided that MlrA displays a second, novel peptidase
214 activity towards this hexapeptide (Dziga et al., 2016). Lastly, and as reiterated in section 3.1, the
215 first evidence for the degradation of Adda via phenylacetate metabolism is presented by Yang
216 et al. (2020). Note that Harada et al. (2004) purified Adda via microbial degradation of MC-LR
217 with B-9 cell extract and demonstrated non-toxicity via mouse bioassay, providing additional
218 evidence that the cyclic MCs structure is primarily responsible for toxic effects.

219 The following two studies described *mlrA*-verified strains that presented *mlr* genomic
220 regions but suggested a lack of functional *mlrB*. Jiang et al. (2011) first described the MlrA
221 verified MCs-degrader *Novosphingobium* sp. strain THN1 (hereafter THN1) that contained MlrB
222 inactivated via a frameshift mutation. Zhang et al. (2017) offered the first description of
223 *Sphingopyxis* sp. strain a7 (hereafter a7), which was capable of degrading MC-LR at 3.33 mg l^{-1}
224 h^{-1}, one of the highest natural degradation rates described. It should be noted that the a7
225 isolate was cultured in the presence of MCs prior to assay, inducing expression of the Mlr
226 pathway. The presence of *mlrACD* (i.e. lacking *mlrB*) was verified via PCR, further suggesting
227 (along with Jiang et al., 2011) that *mlrB* may not be required for complete MCs degradation
228 (Zhang et al., 2017). An excellent comparison of the degradation rates of various bacterial
229 isolates is provided in Table 2 of Zhang et al. (2017).

230 Finally, intriguing and potentially relevant results from studies where *mlrA* is not verified
231 are briefly noted. Manage et al. (2009) presented the first report of MCs-degraders that do not
232 belong to the *Proteobacteria*, instead belonging phylum *Actinobacteria*, with isolates from
233 genus *Rhodococcus*, *Arthrobacter*, and *Brevibacterium* reported, and all isolates testing
234 negative for *mlrABC* via PCR analysis. Despite these negative *mlrABC* PCR results, the observed

MCs-degradation products of *Rhodococcus* sp. C1 were similar to those initially described by Bourne et al. (1996) (Lawton et al., 2011). Zhang et al. (2011) reported a *Ralstonia solanacearum* isolate capable of degrading MC-LR at the rate of 9.4 mg l^{-1} day^{-1}, which was the highest reported rate at this time. Analysis of the degradation products again revealed the linearization via hydrolysis of Arg-Adda bond as a primary step in degradation. A novel secondary degradation product was observed with a re-cyclization of the linear molecule presented as a small peptide ring produced via interaction of the Arg R-group with the adjacent D-(iso)MeAsp residue, no data on *mlr* is given (Zhang et al., 2011).

3. Genetic and biochemical aspects of MlrA

3.1. Complete genome announcements of natural MlrA expressing strains and genetic analyses

The whole genome sequence of *Sphingopyxis* sp. strain C-1 (hereafter C-1), along with the first description of *mlrE* (peptide-modifying dipeptidase) and *mlrF* (D-aminoacylase) was provided by Okano et al. (2015). Jin et al. (2018) presented the complete genome sequence of the B-9 isolate, including *mlrABCDEF.* Wang et al. (2018a) reported comparative whole genomic analysis of strain THN1 (*mlrABCDEF* verified), with 21 additional degradative *Novosphingobium* genomes. The high synteny of the *mlr* gene cluster was emphasized, as the same gene organization (with the actual gene organization being *mlrCADBEF*, with *mlrCB* encoded by the antisense strand) and ≥85% gene sequence identity was observed between the 7 isolates containing *mlr*. This was taken as evidence supporting horizontal gene transfer of the *mlr* genes

as the primary mechanism affecting their communal distribution, even between genera (Wang et al., 2018a). Qin et al. (2019) described isolation of *Sphingopyxis* sp. strain X20 (hereafter X20, *mlrABCDE* verified) and continued to build on the *mlr* gene cluster evolutionary analysis of Zhu et al. (2016) via thermal asymmetric interlaced (TAIL)-PCR of X20, followed by genomic island analysis of X20 and available completely sequenced isolates C-1, B-9, and THN1. Okano et al. (2020) provided the complete genomic sequence of *Novosphingobium* sp. strain MD-1 (hereafter MD-1), with *mlrABCDEF* verified.

Zhang et al. (2020) re-evaluated 16S rRNA based phylogeny of known MCs-degraders, and also performed a complete genomic analysis of Mlr-verified isolates MD-1, B-9, and C-1, with a focus on metabolic potential and ecological ramifications. Genes potentially involved in phenylacetate biodegradation, along with transposable elements, were observed in the genomic neighborhoods of the *mlr* gene cluster (Zhang et al., 2020). In accordance with this, recent results with Mlr-dependent *Sphingopyxis* sp. strain YF1 (hereafter YF1) support Adda degradation via phenylacetate metabolism, with the complete genomic sequence of this strain also provided (Yang et al., 2020). In summary, such complete genomic analyses have clarified Mlr pathway evolution and integration into native central metabolism. Such genomic information is further essential for omics-scale investigations, such as the pioneering global transcriptomic analysis of strain THN1 when degrading MC-LR under different carbon concentrations (Wang et al., 2019, see section 3.3). Future investigations may utilize such state-of-the-art global analyses to clarify the impacts of MlrA overexpression within non-native hosts. Such results may inform optimization strategies for heterologous protein production or offer novel approaches for modulation of primary metabolism.

278

279 **3.2. Degradation of MCs variants**

280 Imanishi et al. (2005) provided a detailed chromatographic description of the
281 degradation products via B-9 cellular extract on multiple MCs variants and nodularin via LC/MS.
282 Interestingly, B-9 was fully capable of degrading nodularin via initiating hydrolysis at the Arg-
283 Adda peptide bond, unlike previously reported for strains ACM-3962 and MD-1. Furthermore,
284 B-9 cellular extract showed minimal degradation of MC-LF, containing Phe-Adda (unlike whole-
285 cell culture of *Sphingomonas* isolate 7CY described by Ishii et al. 2004), 6(Z)-MC-LR, and 6(Z)-
286 MC-RR. Even with the noted the requirement to optimize MS protocols for accurate
287 quantification of degradation products originating from MCs containing more hydrophobic
288 amino acids at the 4^{th} position (Phe in MC-LF and Trp in MC-LW) (Dziga et al., 2012), the above
289 reports suggest variation in the substrate specificities and activity profiles between bacterial
290 isolates employing homologous MlrA peptidase reactions. Such variation may be due to
291 multiple factors, such as: (i) MlrA homolog amino acid sequence variations, (ii) differences in
292 reaction conditions and analytical protocols, and (iii) additional enzymatic functionalities
293 targeting MCs or MCs degradation products that may have not been elucidated. This suggests
294 that heterologous MlrA expression utilizing well characterized expression systems and chassis,
295 along with researched, robust analytical methodologies are fundamental to exploring the actual
296 effects of MlrA amino acid sequence variation during comparative enzymatic characterization.

297 Ho et al. (2012) provided a first description of *Sphingopyxis* sp. strain TT25 (hereafter
298 TT25), with *mlrA* shown to be 99% similar to strain *Sphingopyxis* sp. strain LH21 (hereafter
299 LH21). TT25 was capable of fully degrading MC-LR, -RR, -YR, and -LA (each at 10 μg l^{-1}), even in

300 the presence of the common algaecide copper sulfate (0.5 mg l^{-1} as Cu^{2+}). TT25 degraded all
301 MCs under both conditions (with and without Cu^{2+}) within 2 days, and when these batch
302 cultures were re-spiked with the MCs set, all MCs were degraded within 4 h under both
303 conditions (Ho et al., 2012).

304 Maghsoudi et al. (2016) gave the first description of *Sphingopyxis* sp. strain MB-E
305 (hereafter MB-E, 99% 16S rRNA homology with C-1 isolate), from Missisquoi Bay, Quebec,
306 Canada, and verified the presence of *mlrABCD*, along with the degradation of MC-LR, -YR, -LY, -
307 LW and -LF. Degradation rate constants were provided for each of the MCs, noting that similar
308 biodegradation patterns between the MCs were observed. MB-E was capable of complete
309 simultaneous degradation of all 5 MCs (each present at 10 µg l^{-1}) within 16 h. The induction of
310 *mlrABCD* from (i) MC-LR, and (ii) mixture of the 5 MCs, was also investigated, showing
311 comparable results, supporting the application of this pathway for general MCs degradation.
312 The pH effect on degradation of the 5 MCs was described, continuing observations that near
313 neutral pH allows for optimal growth/degradation (Maghsoudi et al., 2016). Finally, as related
314 information, Kato et al. (2007) provided the first evidence that crude extracts from isolate B-9
315 can degrade other non-MCs cyanobacterial cyclic peptides (microcyclamide, aeruginopeptin 95-
316 A, microviridin I). Table 1 presents the reported MlrA substrates given for each verified strain.

317

318 **3.3. MCs and exogenous nutrient effects on *mlrA* expression in natural isolates and *in situ***
319 **microbial consortia**

Studies highlighted in this section primarily focus on *mlrA* expression within (i) a natural isolated strain, or (ii) *in situ* microbial consortia (this fundamental difference is important to note when comparing between such studies). First, we review studies utilizing individual isolates, followed by reports describing microbial consortia. The first reports of the effect of MC-LR and degradation products on the transcription of *mlrABC* within isolate C-1 demonstrated that cyclic MC-LR induced transcription of *mlrABC* (Shimizu et al., 2009, 2011). *mlrA* was induced during late-log phase growth, while linearized MC-LR and Adda were shown to induce transcription of *mlrAB* but not of *mlrC*. These findings may serve to inform novel approaches for 'priming' industrial biofilters with Adda (or similar non-toxic metabolites that induce MCs biodegradative pathways) for the purpose of reducing the lag phase often associated with *in situ* biodegradation processes during MCs contamination events. Similarly, Jiang et al. (2011) showed *mlrA* is upregulated by MC-LR in the THN1 isolate, although the MC-LR concentrations utilized in this study (400 and 2000 μg l^{-1}) were significantly greater than those most commonly encountered in natural settings.

Further description of C-1 (isolated from Lake Hongfeng in Guizhou Province, China, pH 9.5) noted the alkali tolerance of this strain compared to strain MD-1 (Okano et al., 2009). MD-1 showed growth inhibition at pH 9 and growth arrest at pH 10, while C-1 was capable of growth at pH 11 (although with optimal growth pH 7). Despite the consistent growth rates of C-1 at pH 10 compared to pH 7, MCs degradation showed an optimal pH range of pH 6.72 to pH 8.45. This again suggests that heterologous expression could help to normalize for chassis effects when performing comparative MlrA characterizations.

Utilizing strain THN1, Wang et al. (2019) showed that decreasing the available organic carbon availability (i.e. glucose, starch and sodium pyruvate) resulted in the maximal MC-LR degradation rate occurring earlier in the biodegradation process. Furthermore, MC-LR did not promote the growth of THN1, and MC-LR degradation was related to *mlrA* gene expression, but not to biomass. Thus, THN1 MCs degradation primarily results from the expression of functional MlrA (Wang et al., 2019). These results are consistent with above reports of Shimizu et al. (2009, 2011) (Wang et al., 2019). Finally, Wang et al. (2019) performed a whole genome expression analysis on THN1 during MC-LR degradation at two separate carbon concentrations (100%, 40%). MC-LR degradation led to global transcriptomic effects, including upregulation of glutathione metabolism related genes such as GST. The authors highlight that glutathione metabolism may function in concert with Mlr during MCs degradation. As with Jiang et al. (2011), the results of Wang et al. (2019) must be interpreted considering the high MC-LR concentrations applied (1.5 and 3 mg l^{-1}), which are 2-3 orders of magnitude greater than those commonly observed under natural limnological conditions. Very recently, Zeng et al. (2020) have provided the first evidence that acyl-homoserine lactone mediated quorum sensing acts as a transcriptional activator of the Mlr pathway in a natural strain (*Novosphingobium sp.* ERW19). Quorum sensing systems are present in all completely sequenced genomes of MCs-degraders, and may inform novel methods for *in situ* activation of Mlr in natural populations.

A further study, focused on the regulation of *mlrA* expression in microbial consortia, described a quantitative TaqMan PCR assay and analyzed *mlrA* gene abundance within a sand filter biofilm community during MC-LR degradation (Hoefel et al., 2009). A close association between *mlrA* copy number and MCs-removal, along with re-emphasis of the degradation lag

363 phase upon initiating MCs exposure, was observed. A clonal library was then generated to
364 investigate the diversity of *mlrA* homologs within the sand filter. Sequencing analysis of 50
365 isolates produced three unique *mlrA* gene sequences, each had >98% similarity to previously
366 described *mlrA* sequences. Ho et al. (2010) utilized this TaqMan assay to produce additional
367 data that showed MC-LR (at 20 μg l^{-1}) was directly associated with increased presence of *mlrA*
368 within sand filter biofilms, and demonstrated a >5-fold increase in *mlrA* gene copy number ml^{-1}
369 over an 8-day period.

370 Li et al., (2011a) also showed an increase of *mlrA* copy number upon exposure to MC-LR
371 within a biofilm bacterial community from a Lake Kasumigaua (Japan) water treatment plant. Li
372 et al. (2011b, 2012) further characterized the effects of additional nutrients (i.e. phosphate,
373 glucose, glucose and nitrate, and peptone and ammonium) on both the MC-LR degradation rate
374 and *mlrA* gene copy number in that biofilm community. MC-LR was shown to increase *mlrA*
375 copy number, although co-application of some nutrients (i.e. phosphate, glucose, and peptone
376 and ammonium) reduced this effect and slowed MC-LR degradation. Critically, the co-addition
377 of nitrate was shown to ameliorate the effect of added glucose, allowing complete degradation
378 within 7 days (vs. 10 days). Notably, in the absence of MC-LR nutrient addition did not increase
379 *mlrA* gene copy number (Li et al., 2012). It was further postulated that the presence of
380 exogenous nutrients allows for proliferation of non-MCs degrading bacterial populations,
381 resulting in increased competition and growth inhibition of MCs degraders. Investigating these
382 nutrient effects in an individual isolate, Li et al. (2014) explored the effects of various nutrients
383 (i.e. sodium nitrate, ammonium chloride, and dipotassium phosphate) on *mlrA* expression in
384 the isolated THN1 strain. Also employing the Lake Kasumigaua-sourced biofilm used by Li et al.,

Wang et al. (2016) noted the effect of metal ions (i.e. Mn^{2+}, Zn^{2+} and Cu^{2+}, each at 1 mg l^{-1}) on both MC-LR degradation and qPCR of *mlrA*, with degradation inhibited by Mn^{2+}. Below, we consider reports utilizing bacterial communities isolated directly from HABs.

Lezcano et al. (2016) observed presence of *mlrA* in only about 27% of the most efficient MCs degrading bacteria isolated from a MCs-producing *Microcystis* bloom, indicating the substantial contribution of Mlr-independent bioprocesses. Despite this, bacterial isolates utilizing the *mlr* pathway (given in Table 1) showed higher degradation rates, especially in the presence of alternative C and N sources (Lezcano et al., 2016). Work from Morón-López et al. (2017) further supported the above, with observations that Mlr-independent pathways were less efficient in MCs degradation under all tested conditions (nutrient addition, temperature) compared to Mlr-dependent metabolism, thus strengthening biotechnological implementation of Mlr-dependent approaches. Lezcano et al. (2018) provided an analysis of the dynamics of (i) MCs concentration (both sestonic and dissolved), (ii) chlorophyll a concentration, (iii) water physicochemical properties, (iv) cyanobacterial community composition, (v) qPCR analysis of microcystin biosynthesis gene *mycE*, and (vi) qPCR analysis of *mlrA*, over a 3 year period at the San Juan reservoir in Madrid, Spain. In general, it was observed that there was an increase in *mlrA* gene abundance that followed *mycE* increase (with subsequent *mycE* decline), which supported previous observations of Zhu et al. (2014). Further, MCs degradation capacity was only observed after pre-exposure to MCs, despite the presence of *mlrA* in pre-HAB periods (Lezcano et al., 2018).

In conclusion, the Mlr-based degradative capacity of natural communities is fundamentally dependent on *mlrA* expression, which is under tight control and significantly

upregulated upon exposure to MCs. Additionally, the presence of alternative C and N sources can negatively impact *mlrA* expression. Acyl-homoserine lactone mediated quorum sensing can positively modulate *mlrA* expression, with further investigation into *mlrA* regulation via such quorum sensing systems of great interest. Finally, continued clarification of any relationships between expression/activity of glutathione metabolism related genes (especially including GST, given GST implication in Mlr-independent degradation) and *mlr*-expression is highly relevant. Any impacts of the metabolic substrates/products of these glutathione metabolism related genes should likewise be considered.

3.4. Heterologous expression and biochemical characterization of MlrA

Dziga et al. (2012) provided the original comparative analysis of MlrA activity between natural isolates and heterologous expression in non-native organisms. ACM-3962 MlrA was expressed in both *E. coli* BL21(DE3) and *Staphylococcus aureus* 178RI (Table 2). Expression in *E. coli* resulted in cell extracts showing 6800-fold higher MlrA activity compared to extracts of ACM-3962, and *E. coli* whole cell MlrA activity was 250-fold higher than that in ACM-3962. These results first suggested experimental directions focusing on direct application of MlrA enzyme *in situ*. The following information was also included: (a) the first description of MlrA kinetics, (b) application of a C-terminal His-tag, (c) verification of the previous report by Bourne et al. (1996) that o-phenantroline is a stronger inhibitor than EDTA, (d) mutation within the highly conserved HAIHNE motif render MlrA inactive [with ACM-3962 native MlrA $H^{260}AIH^{263}NE^{265}$, and mutations MlrAH260A and MlrAE265A analyzed], and (e) a protein phosphatase 1 inhibition assay that showed linearized MC-LR had 2100-fold lower inhibition

(toxicity) than MC-LR, significantly less toxic than that reported by Bourne et al. (1996), and attributed to a more complete HPLC product separation during material preparation. Finally, it should be noted that MlrA recovery was reduced in the presence of 1% Triton 100 detergent compared to phosphate-buffered saline (PBS). This, along with the observed ratio of MlrA activity (cell extract:whole cell) compared between the heterologous (ratio = 440) and natural chassis (ratio = 30), thus showed that in the natural chassis MlrA activity is more completely associated with the outer cell compartments, with the authors taking this as evidence to suggest that during heterologous expression MlrA may not be as efficiently localized. This may be a factor in challenges encountered during MlrA overexpression, such as the observed growth arrest upon IPTG induction of MlrA expression (Dziga et al., 2012). Recent results suggest that N-terminal modification via peptide tag application may be an effective approach to such challenge (Dexter et al., 2018; Liu et al., 2020).

Maseda et al. (2012), provided strong evidence that MlrA is the sole enzyme responsible for initiating the *mlr*-dependent degradation pathway. This was shown via knockout of *mlrA* in C-1 via homologous recombination leading to complete loss of cyclic MC-LR degradation, yet with maintained capacity to degrade both linear MC-LR and tetrapeptide. Furthermore, immunoblot analysis of a recombinant C-1 strain that expressed MlrA with a C-terminal His-tag indicated that the tagged MlrA in the native chassis was present entirely within the membrane fraction, with no cytosolic detection (Maseda et al., 2012). The authors stated that MlrA in the native C-1 isolate is believed to be exclusively localized to the inner membrane. For future analysis of MlrA localization, the protocols utilizing intensive centrifugation (200,000 × g at 20°C

for 60 min) for fractionation of cell extracts after sonication should be noted (Maseda et al., 2012).

Zhu et al. (2016) isolated novel *Rhizobium* sp. TH, verified and heterologously expressed *mlrA*, and performed a detailed *mlrA* phylogenetic analysis. Results based on homology and *mlrA*/genomic GC-content supported *mlrA* first occurrence and vertical evolution in α-proteobacteria, followed by horizontal gene transfer to ß- and γ-proteobacteria, as respectively represented by *Bordetella* sp. (from Yang et al. (2014)) and *Stenotrophomonas* (from Chen et al. (2010)) (Zhu et al., 2016). Zhu et al. (2016) also noted that the *Rhizobium* sp. TH *mlrA* homolog is predicted to have a 23 AA N-terminal signal peptide, compared to the 26 AA signal peptide first reported (Bourne et al., 2001) and predicted in *mlrA* homologs from isolates: (i) ACM-3962, (ii) C-1, (iii) *Sphingopyxis* sp. USTB-05 (hereafter USTB-05), and (iv) THN1.

Yan et al. (2012a, 2012b) described the first expression of USTB-05 MlrA in *E. coli* and verified activity towards MC-LR, -RR, with Wang et al. (2013) showing MC-RR, -YR degradation. Xu et al. (2019a) utilized both heterologously expressed USTB-05 MlrA (as described by Yan et al. (2012b)) and MlrC to clarify the nodularin biodegradation pathway. MlrA peptidase activity against nodularin was congruent to previously described mechanisms: hydrolytic peptidase activity via adding one hydrogen on the NH_2 group of Adda and hydroxylation of the carboxyl group of Arg. Interestingly, analysis of 7 site-directed MlrA mutants, focusing on critical amino acids (Table 2) noted that while W176A, W201A, and N264A mutants retained some activity against MC-LR, all 7 mutants completely abolished nodularin degradation (Xu et al., 2019a). Utilizing these same 7 mutants combined with *in silico* molecular dynamics simulations, Xu et al. (2019b) provided the most comprehensive analysis and discussion of the structural basis of

MlrA activity. It was computationally determined that the $H^{260}AIH^{263}NE^{265}$ motif does not sterically facilitate the functioning of Glu^{265} as a catalytic base, as is observed in the HEXXH metalloprotease motif. Additional results noted (i) the conservation of three active site motifs homologous to *Methanococcus maripaludis* glutamate intramembrane protease Rce1, and (ii) comparable resistance to enzyme inhibition via EDTA reported for these two enzymes. These three results lead the authors to conclude that MlrA is likely not a metalloprotease, does not require metal for catalysis, and that MlrA is a glutamate intramembrane protease member of the type II CAAX prenyl endopeptidases with catalytic cavity open to the cytoplasm. Further, the authors provided a proposed enzymatic mechanism: Glu^{172} and His^{205} activate a water molecule facilitating nucleophilic attack on the Arg-Adda peptide bond, Trp^{176} and Trp^{201} contact the carboxylate side chain of Glu^{172} and, by raising its pKa potentially, accelerate the reaction rates, with His^{260} and Asn^{264} (of previously postulated active center of $H^{260}AIH^{263}NE^{265}$) functioning as an oxyanion hole to stabilize the transition states (Xu et al., 2019b). Counterevidence was considered (i.e. potentially supporting the designation of MlrA as a metalloprotease), indicating that if $H^{260}AIH^{263}NE^{265}$ were to be a component of a metal binding network, then a third remote ligand would be required to complete the metal-binding site. Appropriate amino acids are present within MlrA, and Dziga et al. (2012) previously suggested that MlrA may exist in a dimeric form. Xu et al. (2020) successfully utilized the N-terminal GST-tag to purify USTB-05 MlrA from *E. coli* (strain from Yan et al. (2012b)). With successful removal of the GST-tag, purified MlrA was observed at ~ 25 kDa via SDS-PAGE (not the expected 36 kDa), in agreement with this original observation from Dziga et al. (2012). Application of 60 mg l^{-1} of this purified USTB-05 MlrA completely degraded 20 mg l^{-1} of MC-LR (within 10 minutes) and 25

494 mg l^{-1} of nodularin (within 12 hours) (Xu et al., 2020). In conclusion, heterologous MlrA
495 expression has thus offered unprecedented insight into the biochemical properties of this
496 unique enzyme.

497

498 **4. MlrA application and technological development**

499 **4.1. Natural strains**

500 Tsuji et al. (2006) and the US patent US20060096915A1 by Sumino et al. (2006) (Japan
501 Foreign Application Priority Date of Nov 11, 2004) offered the first direct reports highlighting
502 the industrial application of natural MCs-degrading bacterial isolates. Patent US20060096915A1
503 described *Sphingomonas* strain MDB1 (also referred to as strain FERM P-19480) and three
504 industrially-focused methodologies for enabling contact between this strain and MCs
505 contaminated waters: (i) direct surface spraying of MDB1 cultures, (ii) immobilization via
506 entrapment of MDB1, and (iii) immobilization via surface attachment of MDB1. Strain MDB1
507 was described as capable of degrading MC-LR, -RR, -YR, with a cell concentration of 4e8 cells ml^{-1}
508 shown to degrade 100 µg l^{-1} MCs to below 1 µg l^{-1} within 30 h. Direct spraying of cell
509 concentrations between 10^6 - 10^{10} cells m^{-2} surface resulted in MCs-degrading efficacy
510 dependent on applied cell density. In contrast to this, MDB1 immobilization (via entrapment in
511 acrylamide or polyethylene glycol diacrylate) showed significant advantages in terms of
512 efficiency of degradation per cellular biomass applied. Higher MCs degradation rates (µg MCs l^{-1}
513 pellets h^{-1}) were observed for entrapped MDB1 compared to immobilization on carrier surface,
514 although there is no control data given for non-specific MCs adsorption with the immobilization

matrix. Tsuji et al. (2006) described successful surface immobilization of the B-9 isolate using a polyester carrier, which showed a greater than 80% MC-RR removal efficiency over 2 months of continuous bioreactor operation. Phujomjai et al. (2016) verified *mlrABCD* in novel isolate *Novosphingobium* sp. KKU25s (isolated from the Bueng Nong Khot reservoir in Khon Kaen, Thailand), and showed a [Dha^{7}]MC-LR (representing dehydroalanine (Dha) substituting for the more commonly observed methyldehydroalanine at the 7th position amino acid of the MC-LR heptapeptide ring) degradation rate of 0.7 μg l^{-1} h^{-1} utilizing cells immobilized on a plastic carrier. Wu et al. (2020, note corrigendum Wu et al., 2019b) and Ren et al. (2020) investigated whole cell immobilization of isolate YF1. Wu et al. (2020) described immobilization on glutaraldehyde-activated chitosan-modified magnetic Fe_3O_4 particles, while Ren et al. (2020) utilized activated carbon fibers as a carrier with immobilization via sodium alginate. Comparison between these two immobilization methods is complicated by the different MCs substrates utilized in each study, with Wu et al. (2020) reported a MC-LR degradation rate of 1.50 μg ml^{-1} h^{-1}, while Ren et al. (2020) described MC-RR degradation at 0.76 μg ml^{-1} h^{-1}.

Ho et al. (2006) provided the first PCR verification of *mlrA* isolated from a biologically active sand filter utilized for MC-LR, -LA degradation. A 3-day lag phase before initiation of degradation was observed, yet once degradative activity was established that even operating conditions approximating fast sand filtration were sufficient for toxin removal, given greater biomass at the top portion of the sand column. Recent results by Jeon et al. (2020) further demonstrated this accelerated MC-LR degradation (with increased *mlrA* gene copy number) in the upper layers of biologically active filters. Additional verification for MCs biodegradation within sand filters was done by Ho et al. (2007b) considering various sand/water sources, initial

MC-LR concentrations, and operating temperature, which was noted as a potential limiting factor due to significantly reduced biodegradation at 10°C compared to 20°C. Bourne et al. (2006) offered concurrent results describing direct application of strain ACM-3962 to contaminated waters and the first description of application of this isolate to biologically active slow sand filters. Results clearly indicated that ACM-3962 applied to flask liquid cultures at any of the examined cell densities resulted in accelerated MC-LR degradation. Results describing the inoculation of the slow sand filters were less conclusive, showing no enhanced MC-LR degradation with ACM-3962 inoculation, most likely due to experimental design (Bourne et al. 2006). More recently, Morón-López and Molina (2020) demonstrated the recycling of end-of-life reverse osmosis membrane as attachment substrate for a biofilm-based bioreactor utilizing MlrA-verified strain *Sphingopyxis* sp. IM-1. In a complimentary study, 3 types of such recycled reverse osmosis membranes were applied to natural cyanobacterial bloom (San Juan reservoir), allowing membrane colonization and biofilm formation directly via environmental MCs-degrading communities (both Mlr-dependent and Mlr-independent), with the presence of *mlrA* confirmed in all such membrane-based consortia (Morón-López et al., 2020).

Wang et al. (2018b) demonstrated that direct application of cultured *mlrA* containing natural isolates to microcosm constructed wetlands markedly enhanced MC-LR degradation and allowed for degradation of 16.7 μg l^{-1} MC-LR to below 1 μg l^{-1} within 12 h, compared to 72 h for the control. Lee et al. (2018) isolated a *Bacillus* sp. T4 (hereafter T4) that showed lethal activity towards *M. aeruginosa*, demonstrated that following application and *M. aeruginosa* lysis that extracellular MCs concentration was dramatically increased. Lee et al. (2018) thus

558 further explored the novel co-application of both T4 (as cyanobactericide) and *mlrA*-containing
559 isolates (3 isolates individually) for the rapid degradation of released MCs.

560

561 **4.2. Heterologous MlrA expression**

562 Dziga et al. (2013b, 2014) provided the first report of immobilization of previously
563 described *E. coli* expressing MlrA (Dziga et al., 2012) in alginate beads, for use as a whole cell
564 biocatalyst. Both closed and open (flow through column) bioreactors were analyzed, with
565 description provided of (i) activity and immobilization efficiency of alginate beads vs. cell
566 concentration, (ii) column length vs. MC-LR degradation efficiency, and (iii) temporal stability of
567 activity at various temperatures. While the results showed maximal degradation rate per liter of
568 carrier of 219.9 μg MC-LR h^{-1}, a 65% decline in MlrA activity after 72 h of exposure to
569 continuous flow of lake water was observed (Dziga et al., 2014).

570 Wang et al. (2017) presented the first description of heterologous THN1 *mlrA*
571 expression. In contrast to results provided by Dziga et al. (2012), the whole cell recombinant
572 biocatalyst was shown be more efficient than the crude cellular extract, with a maximal MC-RR
573 degradation rate of 9.22 μg ml^{-1} h^{-1} for the first 8 hours of incubation (Wang et al., 2017). See
574 also Li et al. (2018) for THN1 MlrA peptidase verification.

575 The first description of MlrA expression in a photoautotroph utilized model
576 cyanobacterial chassis *Synechocystis* sp. PCC 6803 (Dexter et al., 2018). Application of a 23 AA
577 N-terminal secretion peptide from the native PilA gene (*sll1694*) allowed for stable homologous
578 recombination and expression of USTB-05 MlrA via the strong $P_{cpcB560}$ promoter. The resultant

579 whole cell biocatalyst showed about 3 times higher degradative activity towards MC-LR
580 compared to natural *mlrA* gene host ACM-3962. Also, compared to the *E. coli* chassis, the
581 cyanobacterial chassis demonstrated increased stability of both MlrA activity and biomass over
582 prolonged incubation under semi-natural conditions (lake water). Finally, use of such
583 photoautotrophic chassis may allow for production of MlrA-active cellular extracts at lower
584 costs than production in a heterotrophic chassis, given the reduced input/substrate
585 requirements of photoautotrophic culturing (Dexter et al., 2018, WO2018017828A1), with
586 Dziga et al. (2018) noted as the first study supporting the direct application of such MlrA
587 extracts for the rapid elimination of MCs from the environment. It has been demonstrated that
588 co-application of heterologously produced MlrA (*E. coli* BL21(DE3) cellular extracts) with H_2O_2
589 allowed for significantly accelerated MCs clearance following HAB lysis resulting from the
590 cyanobactericidal action of H_2O_2 (Dziga et al., 2018). It was also noted that MlrA activity
591 remained unaffected even at H_2O_2 concentrations 100-fold greater than those recommended
592 for cyanobactericide. This research indicated that direct application of heterologous MlrA
593 extracts seemed to be much more efficient at MCs-decontamination compared to use of whole
594 cell biocatalysts – either as natural strains or use of immobilized whole cell *E. coli* MlrA
595 expression chassis (as from Dziga et al., 2014).

596 The above research of direct co-application of H_2O_2 and MlrA has been extended for
597 simultaneous *in situ* reduction of both HAB cyanobacterial biomass and the resultant
598 extracellular MCs via mesocosm experiments in highly eutrophic Lake Ludoš, Serbia (Dziga et
599 al., 2019). Results indicated that (i) environmental pH and temperature influence MlrA activity,
600 (ii) for experimental conditions of 20 °C, pH 7.0-9.5, 5 mg l^{-1} of H_2O_2, 14 Units of MlrA (per 100 l

601 contaminated with 10 µg MCs l^{-1}) were found to efficiently eliminate both cyanobacteria and
602 MCs in lake water, (iii) novel *E. coli* C41 (DE3) chassis (with previously described pET21a-mlrA
603 expression vector) allowed for more efficient MlrA production, and (iv) MlrA co-application
604 with H_2O_2 resulted in accelerated MCs detoxification *in situ* (Dziga et al., 2019). The need for
605 further experimentation was underscored, regarding any stimulating effect of MlrA extracts on
606 subsequent cyanobacterial re-growth (possibly due to nutrient addition present within the
607 crude cellular MlrA extracts) and also the effects of H_2O_2 application on gene expression of the
608 MCs biosynthesis gene cluster (potential upregulation, even with significant reduction in both
609 total cellular biomass and thus intracellular MCs load) (Dziga et al., 2019).

610 Wu et al. (2019a) expressed *Sphingopyxis* sp. M7 MlrA in *E. coli*, and then provided the
611 first description of MlrA enzyme immobilization, utilizing L-cysteine modified graphene oxide.
612 Activity of immobilized MlrA was compared to free MlrA for nodularin degradation, key findings
613 included: (i) both immobilized and free MlrA demonstrated similar degradation efficiency, (ii)
614 degradative efficiency of immobilized MlrA was greater than 81% of the initial efficiency after 7
615 times repeated use (involving washing/drying of immobilized graphene oxide-MlrA
616 nanocomposite), and (iii) immobilized MlrA activity showed superior stability during storage at
617 0 °C (Wu et al., 2019a). Finally, China patent CN103555696B (filed Nov 6, 2013) showed
618 successful application of the N-terminal maltose binding protein (MBP, from the pMAL-c2X
619 plasmid) that allowed for efficient MlrA purification from *E. coli*. Liu et al. (2020) utilized this
620 same MBP to allow for increased solubilization, overexpression, and purification of the C-1 MlrA
621 homolog. MlrA activity was determined for purified MlrA both with the tag (MBP-MlrA) and
622 following tag removal, the MBP-MlrA showed superior MlrA activity (Liu et al., 2020). Further

623 results indicated (i) first report of successful chromatographic purification of MBP-MlrA to >
624 90% purity, (ii) kinetic comparison between crude cell extracts and purified enzyme, the
625 purified enzyme showed significantly increased catalytic constant (K_{cat}), (iii) thermal, pH, and
626 half-life at room temperature stability analyses indicated that purified MBP-MlrA demonstrated
627 high stability, (iv) application of purified MBP-MlrA showed both detoxification of extracellular
628 MC-LR and inhibition of *Microcystis aeruginosa* strain FACHB 905, via downregulation of MCs-
629 biosynthesis pathway and photosynthesis gene expression (v) inhibition via MBP-MlrA was
630 selective to MCs-producer *M. aeruginosa* strain FACHB 905 and not observed in *Synechocystis*
631 sp. PCC 6803 cultures, and (vi) MBP-MlrA showed no toxicity towards human cells (Liu et al.,
632 2020). In summary, the above results of Liu et al. (2020) are the first description of using a
633 single enzyme for simultaneous killing of toxigenic cyanobacteria and toxin degradation, along
634 with broad enzymatic analysis that strongly supports MlrA technological potential. In the next
635 section, we resynthesize the above information for deeper discussion, with the goals of
636 developing understanding and potential future research trajectories.

637

638 **5. Discussion and potential future directions**

639 Substantial progress has been made in the 25 years since the first report of MlrA
640 function, which have broadly helped to inform on the distribution, role and properties of this
641 enzyme within both the natural environment and industrial contexts. This section focuses on
642 potential applications of MlrA activity as a sustainable biotechnological product. As a general
643 overview (and as shown in Figure 3), MlrA-based technologies can be considered from the
644 following trajectories: (i) direct modulation of Mlr activity within naturally existing populations,

(ii) bioaugmentation of natural/engineered systems with introduced biodegradative capacity via whole cell biocatalysts (either native/natural MlrA chassis or heterologous chassis), or (iii) MCs bioremediation via direct MlrA application, with considerations regarding biocatalyst immobilization/localization relevant mainly to points (ii) and (iii), but also possibly to point (i).

5.1. Direct modulation of Mlr activity within naturally existing populations

Studies reviewed in section 3.3, along with the work of Morón-López et al. (2020), Jeon et al. (2020), Somedee et al. (2013), and Ho et al. (2006, 2007a, 2007b, 2012) are fundamental to understanding the potential for modulation of environmental MlrA dynamics. In addition to these reports, recent work (Silva et al., 2018, 2019; Silva and Pernthaler, 2019; Kumar et al., 2018, 2019a) must be considered, given relevance to natural MCs-degrading populations. Silvia et al. (2018) described the 'priming' effect via addition of inactivated *Microcystis* sp. biomass (both toxic and non-toxic, MCs-free mutant) to gravity driven membrane filtration biofilms, for MCs-degradation. It was observed that the non-toxic biomass comparably primed MCs-degradation capacity (Silva et al., 2018), with Silva and Pernthaler (2019) determined that this priming effect was due to promotion of oxygen-limited conditions via substrate (biomass) addition, with such anaerobic conditions stimulating facultative MCs-degrading communities. Critically, these two reports noted that MCs-degradation within the membrane biofilm system was mainly due to anaerobic processes (Mlr independent). Additionally, significantly higher MCs-degradation was observed in biofilm fed with water experiencing regular HAB (lake) when compared to stream water, yet both biofilms demonstrated comparable MCs-degradation capacity during enrichment culturing on MC-LR (Silva et al., 2019). Microbial community

667 analysis (16S rRNA) performed on the gravity driven membranes, followed by co-occurrence
668 network analysis indicated that lake communities showed reduced genotype interlinkage and
669 thus greater stochasticity in community assembly that was proposed to facilitate the
670 establishment of facultative MCs-degraders *in situ* (Silva et al. 2019). Kumar et al. (2018)
671 showed that MC-LR enriched bacterial communities isolated from various units of a drinking
672 water treatment plant (raw water/pre-ozonation, effluent sludge sedimentation unit, and top-
673 sand filtration unit) gave enhanced MC-LR degradation rates (1.5-fold) compared to non-MC-LR
674 acclimatized communities. Finally, the primary challenge in direct modulation of Mlr activity
675 within naturally existing populations has been emphasized by Kumar et al. (2019a): the lag
676 phase (often days in duration) occurring before industrially relevant MCs degradative activity is
677 achieved. This needs to be addressed to enable maximal MCs degradation immediately
678 following environmental MCs loading events. Opportunities for combination of methodologies
679 described in section 5.1-5.3 should be noted. For example, direct application of MlrA during this
680 lag phase may provide the rapid response required to establish industrially robust biofilm-
681 based treatments. Also, novel combination with existing physicochemical treatments may
682 further extend any application scope.

683

684 **5.2. Bioaugmentation with whole-cell biocatalysts**

685 Work reviewed in section 4.1, along with the work of Wang et al. (2017), Dexter et al.
686 (2018), and Dziga et al. (2013b, 2014, 2018) are relevant to application of whole-cell MlrA
687 expressing biocatalysts *in situ*. In addition, Kumar et al. (2019b) described the application of
688 known MCs degraders to native bacterial communities at a water treatment plant and verified

689 successful enhancement of MCs-degradation with *Sphingomonas* and *Arthrobacter*. Several
690 considerations regarding this approach should be noted. First, when considering application of
691 a whole-cell biocatalyst expressing MlrA, successful commercial application of heterologous
692 MlrA-expressing chassis is much less likely than use of native strains naturally containing MlrA.
693 Primary reasons may include regulatory restrictions on the environmental application of viable
694 genetically modified organisms and also the fact that model organisms utilized for heterologous
695 expression typically are poorly suited for growth under natural conditions, often rapidly
696 outcompeted by locally established communities. On the other hand, genetically modified
697 organisms with significantly enhanced MCs degradation rate (such as the 250-fold greater
698 activity over natural MlrA gene host ACM-3962, Dziga et al., 2012) may be an alternative in
699 some circumstances, when urgent MCs elimination is of crucial importance. Given this, it is
700 suggested that future endeavors focused on bioaugmentation via the application of viable
701 MlrA-expressing cells, focus on the use of natural isolates, ideally using native, local isolates.
702 Secondly, considerations regarding application of free vs. immobilized whole-cell biocatalyst is
703 of fundamental importance, as presented by Kumar et al. (2019a), with a strong case made for
704 the beneficial effects of immobilization throughout the literature. Thirdly, accurate comparison
705 of whole-cell based biocatalysts will be greatly benefited by agreement on suitable laboratory
706 conditions approximating applied environments. Such conditions should be as essential as
707 possible, enabling widespread, yet accurate implementation, facilitating comparative analysis.
708 An example of such comparative analysis would be as given by Ho et al. (2007a), where isolate
709 LH21 was directly compared to ACM-3962, where LH21 showed more rapid degradation of MC-
710 LR, -LA under equivalent conditions. Specific examples of laboratory conditions that require

711 discussion and standardization may be, (a) degradation experiments utilizing MCs at
712 environmentally relevant concentrations (5-10 μg l^{-1} suggested), (b) incubation of pre-
713 experimental cultures with MCs, for the purpose of acclimatization (1-3 days suggested), as
714 MlrA expression is directly modulated by the presence of MCs, it is suggested that a common
715 MCs concentration and pre-incubation time is agreed upon that has environmental relevance,
716 and (c) as noted by Manheim et al. (2019), the critical nature of the initial substrate
717 concentration to biomass ratio (S_0/X_0). Comparative analysis of methods under environmentally
718 relevant MCs concentrations cannot be over emphasized, given MlrA degradation kinetics and
719 hazardous MCs concentrations most commonly encountered. Quantification of target
720 biocatalytic biomass may be more challenging under immobilized conditions or within bacterial
721 communities. Also, when considering immobilization, environmental impact of carrier material
722 and increased cost of production of immobilized biocatalyst must be considered.

723

724 **5.3. Bioremediation via direct MlrA application**

725 Section 4.2 provides relevant review, compared to the previous two technological
726 directions, recombinant MlrA offers many unique opportunities in the fields of applied genetics,
727 synthetic biology, and protein engineering. First, it is useful to clarify enzymatic differences
728 between various MlrA peptide sequences, with comparative analysis of MlrAs within a given
729 model chassis thus allowing for normalization of chassis effects. Secondly, there is a significant
730 opportunity for engineering MlrA peptide sequence for exploring the effect of amino acid
731 sequence (including N- and C- terminal tagging strategies) on MlrA activity, specificity,
732 localization, and robustness under applied conditions. This is due to the following: (a)

differences in MlrA localization observed between natural vs. heterologous expression (first noted by Dziga et al. (2012a)), whereby expression in *E. coli* results in significantly higher observed MlrA activity within the cytoplasm, (b) Maseda et al., (2012) showing no MlrA activity within the cytosol of the natural C-1 isolate, (c) the rapid genetic instability observed by Dexter et al. (2018) when trying to express the native USTB-05 MlrA (336 AA) in model cyanobacteria *Synechocystis* sp. PCC 6803, compared to the long-term genetic stability and successful expression with the addition of the N-terminus signal peptide that unexpectedly did not function in a signaling capacity, and (d) both CN103555696B and the work of Liu et al. (2020) that also clearly demonstrated multiple benefits to the application of an N-terminal tag (MBP tag). This knowledge can then directly inform on peptide sequences and expression methods for optimal overexpression within a given chassis. Higher MlrA activity yields might be achieved via a number of mechanisms, such as increased enzyme solubility or successful protein localization/secretion, resulting in reduced impact on chassis physiology. Further, as has already been demonstrated, heterologous MlrA expression provides excellent opportunity for investigation into downstream processing (post MlrA production), for the purpose of developing methodologies towards optimal industrial implementation. Thirdly, the demonstrated potential of MlrA (as a single enzyme) coupled with successful expression in multiple heterologous hosts presents a unique model system for integration with state-of-the-art synthetic biology efforts, with prospective increases in results impact. Finally, there is opportunity for further chassis characterization itself, via the comparison of MlrA expression across multiple gene hosts, with successful MlrA expression described in *E. coli*, *Staphylococcus aureus* 178RI, *Synechocystis* sp. PCC 6803, with Broman et al. (2017) presented evidence that

Saccharomyces cerevisiae can be successfully utilized, and with Dziga noting successful expression via *Pichia pastoris* (unpublished results). Each chassis presents a distinctive system for MlrA expression, with varied substrate and process requirements for optimal MlrA biosynthesis/yield, offering multifaceted opportunities for exploration.

6. Conclusions

MlrA peptidase activity is currently understood to be both the most temporally efficient enzymatic process and the most specific catalyst for all known MCs-degradation pathways. In addition, MlrA and the Mlr pathway are presently the only enzymatic processes with clear genetic and biochemical descriptions available, greatly facilitating modern applied genetics for any relevant technological development. As such, there is strong support for not simply the ecological increase of *mlrA* gene copy number (via natural isolate bioaugmentation), but also the increase in *functional* MlrA activity *in situ*, via above approaches outlined in section 5. Finally, comparative/standardized analytical procedures applied to various MCs-biodegradation methods, allowing for determination of the most promising technological directions, should begin to be considered during this relatively early-phase of biotechnological development. This will benefit the field as a whole by accelerating overall development, maximizing the utility of modern information dissemination and position the field for comparison with established commercial/industrial MCs treatment options. A primary goal for the authors is cultivation of such a collaborative space.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

The research of Dziga described herein was supported by the National Science Centre, Poland (grant numbers UMO-2013/11/B/NZ9/00114 and UMO-2014/15/B/NZ9/04727).

Authors' contributions

JD conceived of the review and wrote the manuscript. AJM critically revised the manuscript. PF critically revised the manuscript. DD contributed to the preparation of the manuscript, including Figures, and critical revision. All authors have read and approved the final manuscript.

Declarations of interest

J. Dexter has a patent pending (WO2018017828A1). All other authors declare no conflict of interest.

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Table 1 (Revised)

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Table 1: Isolated bacterial species that have an *mlr* operon and/or contain a homolog of *mlrA* verified by genetic methods, with cyanopeptides (including MCs) experimentally verified to be degraded.

Strain		Affiliation	*mlr* genes	Investigated Cyanopeptides	Isolation Source	Reference
Sphingomonas sp.	ACM-3962/ MJ-PV	α-proteobacteria	ABCD	MC-LR, MC-RR	Murrumbidgee River, Australia	Jones et al. (1994) Bourne et al. (1996)
Sphingomonas sp.	NV-3	α-proteobacteria	ABCD	MC-LR, [Dha^{7}]MC-LR	Lake Rotoiti, New Zealand	Somdee et al. (2013)
Sphingosinicella microcystinivorans	B-9	α-proteobacteria	ABCDEF	MC-LR, MC-RR, 3-DMMCLR, DHMCLR, MCLR-Cys, Nodularin, microcyclamide, aeruginopeptin 95-A, microviridin I	Lake Tsukui, Japan	Harada et al. (2004) Imanishi et al. (2005) Kato et al. (2007) Jin et al. (2018)
Sphingosinicella microcystinivorans	Y2	α-proteobacteria	ABCD	MC-LR, MC-RR, MC-YR	Extracellular mucilage of *Microcystis* colony, Lake Suwa, Japan	Park et al. (2001) Maruyama et al. (2003) Maruyama et al. (2006) Lezcano et al. (2016)
Sphingosinicella sp.	JEZ-8L	α-proteobacteria	ABCD	MC-LR	Jeziorsko reservoir, Poland	Nájera et al. (2017)
Sphingopyxis sp.	a7	α-proteobacteria	ACD	MC-LR	Lake Taihu, China	Zhang et al. (2017)
Sphingopyxis sp.	C-1	α-proteobacteria	ABCDEF	MC-LR, MC-RR, MC-YR	Lake Hongfeng, China	Okano et al. (2009) Okano et al. (2015) Liu et al. (2020)
Sphingopyxis sp.	IM-1	α-proteobacteria	ABCD	MC-LR, MC-RR	San Juan dam, Madrid, Spain	Lezcano et al. (2016)
Sphingopyxis sp.	IM-2	α-proteobacteria	ABCD	MC-LR, MC-RR	San Juan dam, Madrid, Spain	Lezcano et al. (2016)
Sphingopyxis sp.	IM-3	α-proteobacteria	ABCD	MC-LR, MC-RR	San Juan dam, Madrid, Spain	Lezcano et al. (2016)
Sphingopyxis sp.	LH21	α-proteobacteria	ABCD	MC-LR, MC-LA	Biological sand filter, Australia	Ho et al. (2007)
Sphingopyxis sp.	m6	α-proteobacteria	ABCD	MC-LR	Cyanobacteria salvage yards in Fudu bay, Taihu Lake, China	Ding et al. (2018)
Sphingopyxis sp.	MB-E	α-proteobacteria	ABCD	MC-LR, MC-LW, MC-YR, MC-LY, MC-LF	Lake Champlain, Canada	Maghsoudi et al. (2016)
Sphingopyxis sp.	TT25	α-proteobacteria	A	MC-LR, MC-RR, MC-YR, MC-LA	Myponga Reservoir, Australia	Ho et al. (2012)
Sphingopyxis sp.	USTB-05	α-proteobacteria	ABC	MC-LR, MC-RR, MC-YR, Nodularin	Sediment, Lake Dianchi, China	Zhang et al. (2010) Yan et al. (2012a, b) Wang et al. (2013) Xu et al. (2020)
Sphingopyxis sp.	X20	α-proteobacteria	ABCDE	MC-LR	Sediment, Lake Dianchi, China	Qin et al. (2019)
Sphingopyxis sp.	YF1	α-proteobacteria	ABCD	MC-LR	Lake Taihu, China	Yang et al. (2020)
Sphingopyxis sp.	M7	α-proteobacteria	A	MC-LR, Nodularin	Lake Taihu, China	Wu et al. (2019a)
Stenotrophomonas sp.	EMS	γ-proteobacteria	A	MC-LR, MC-RR	Lake Taihu, China	Chen et al. (2010)
Stenotrophomonas maltophilia	4B4	γ-proteobacteria	ABCD	MC-LR, MC-RR, MC-LW, MC-LF, Nodularin	Beira Lake, Sri Lanka	Idroos et al. (2017)

Stenotrophomonas maltophilia	AR14	γ-proteobacteria	A	Not Given	Kalugumalai, India	Jagadeesan et al. (2015)
Novosphingobium sp.	MD-1	α-proteobacteria	ABCDEF	MC-LR, MC-RR, MC-YR	Biofilm of Water Treatment Facility, Lake Kasumigaura, Japan	Saito et al. (2003) Okano et al. (2020)
Novosphingobium sp.	THN1	α-proteobacteria	ABCDEF	MC-LR	Lake Taihu, China	Jiang et al. (2011) Wang et al. (2018a)
Novosphingobium sp.	KKU25s	α-proteobacteria	ABCD	[Dha^{7}]MC-LR	Bueng Nong Khot reservoir, Khon Kaen, Thailand	Phujomjai et al. (2016)
Novosphingobium sp.	ERW19	α-proteobacteria	ABCDEF	MC-LR, MC-RR	Lake Taihu, China	Zeng et al. (2020)
Novosphingobium sp.	ERN07	α-proteobacteria	ABCDEF	MC-LR, MC-RR	Lake Taihu, China	Zeng et al. (2020)
Rhizobium sp.	TH	α-proteobacteria	ABCD	MC-LR	Lake Taihu, China	Zhu et al. (2016)
Bordetella sp.	MC-LTH1	β-proteobacteria	A	MC-LR, MC-RR	Lake Taihu, China	Yang et al. (2014)
Bacillus sp.	EMB	Bacilli	A	MC-LR, MC-RR	Algal heap, Hudai town, Wuxi, China	Hu et al. (2012)
Bacillus sp.	AMRI-03	Bacilli	A	MC-RR	Tendaha Lake, Saudi Arabia	Alamri (2010)
Bacillus flexus	SSZ01	Bacilli	A	MC-RR	Tendaha Lake, Saudi Arabia	Alamri (2012)
Bacillus licheniformis	PM1	Bacilli	A	Not Given	Utter Pradesh, India	Gandhi and Kumar (2016)

Table 2

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Table 2: MlrA Heterologous Expression Systems

MlrA Homolog		Expression Host	Expression Vector	Additional Notes	References
Sphingomonas sp.	ACM-3962/ MJ-PV	*E. coli* XL-1 blue	pPL-lambda		Bourne et al. (2001)
Sphingomonas sp.	ACM-3962/ MJ-PV	*E. coli* BL21(DE3)	pET21a	C-terminal His-tag used. Mutations investigated: H260A, E265A.	Dziga et al. (2012)
Sphingomonas sp.	ACM-3962/ MJ-PV	*S. aureus* 178RI	pG164	C-terminal His-tag used.	Dziga et al. (2012)
Sphingomonas sp.	ACM-3962/ MJ-PV	*E. coli* C41(DE3)	pET21a	*E. coli* C41(DE3) showed superior MlrA production compared to *E. coli* BL21(DE3).	Dziga et al. (2019)
Sphingomonas sp.	ACM-3962/ MJ-PV	*E. coli* K12 TB1	pMAL-c2X	N-terminal MBP-tag used. MlrA homolog sequence given in SEQ ID NO.1.	CN103555696B (2013)
Sphingomonas sp.	ACM-3962/ MJ-PV	*S. cerevisiae* strain SS328-leu	Not specified		Broman et al. (2017)
Rhizobium sp.	TH	*E. coli* BL21(DE3)	pET32a		Zhu et al. (2016)
Sphingopyxis sp.	USTB-05	*E. coli* DH5α	pGEX-4T-1	tac promoter used. N-terminal GST-tag used.	Yan et al. (2012a) Wang et al. (2013)
Sphingopyxis sp.	USTB-05	*E. coli* BL21(DE3)	pGEX-4T-1	tac promoter used. N-terminal GST-tag used. Xu et al. (2019a) mutations investigated: E172A, W176A, W201A, H205A, H260A, N264A, and E265A.	Yan et al. (2012b) Xu et al. (2019a) Xu et al. (2020)
Sphingopyxis sp.	USTB-05	*Synechocystis* sp. PCC 6803	p6803mlrAsec+: double homologous recombination at host *slr0271* genomic locus	23 AA N-terminal PilA tag enabled genetic stability (vs. no tag).	Dexter et al. (2018) WO2018017828A1 (2018)
Sphingopyxis sp.	C-1	*E. coli* K12 TB1	pMAL-c2X	N-terminal Maltose Binding Protein (MBP)-tagged MlrA showed superior activity, along with other favorable properties.	Liu et al. (2020)
Novosphingobium sp.	THN1	*E. coli* BL21	pET-29a(+)		Wang et al. (2017) Li et al. (2018)
Sphingopyxis sp.	M7	*E. coli*	Not given	MlrA immobilized to L-cysteine modified graphene oxide.	Wu et al. (2019a)
Sphingopyxis sp.	USTB-05	*E. coli* BL21(DE3)	pGEX-4T	Synthesized *mlrA* sequence of USTB-05 N-terminal GST-tag.	Performed in Dziga's laboratory (JU, Kraków), between 2014-17, not published. The expression systems explored to find the efficient method of MlrA purification, but without a substantial improvement in overexpression or purification efficiency.
Sphingopyxis sp.	USTB-05	*E.coli* DH5α	pET59 Dest	Synthesized *mlrA* sequence of USTB-05 Thioredoxin (Trx)-tag.	
Sphingopyxis sp.	USTB-05	*E.coli* DH5α	p5e	Synthesized *mlrA* sequence of USTB-05 Maltose binding protein-tag.	
Sphingopyxis sp.	USTB-05	*E. coli* BL21(DE3)	pGEX-4T	Synthesized *mlrA* sequence of USTB-05 N-terminal combined GST-His-tags.	
Sphingopyxis sp.	USTB-05	*P. pastoris* GS 115	pPIC 9	Synthesized *mlrA* sequence of USTB-05 N-terminal His-tag.	

Figure 1 (Revised)

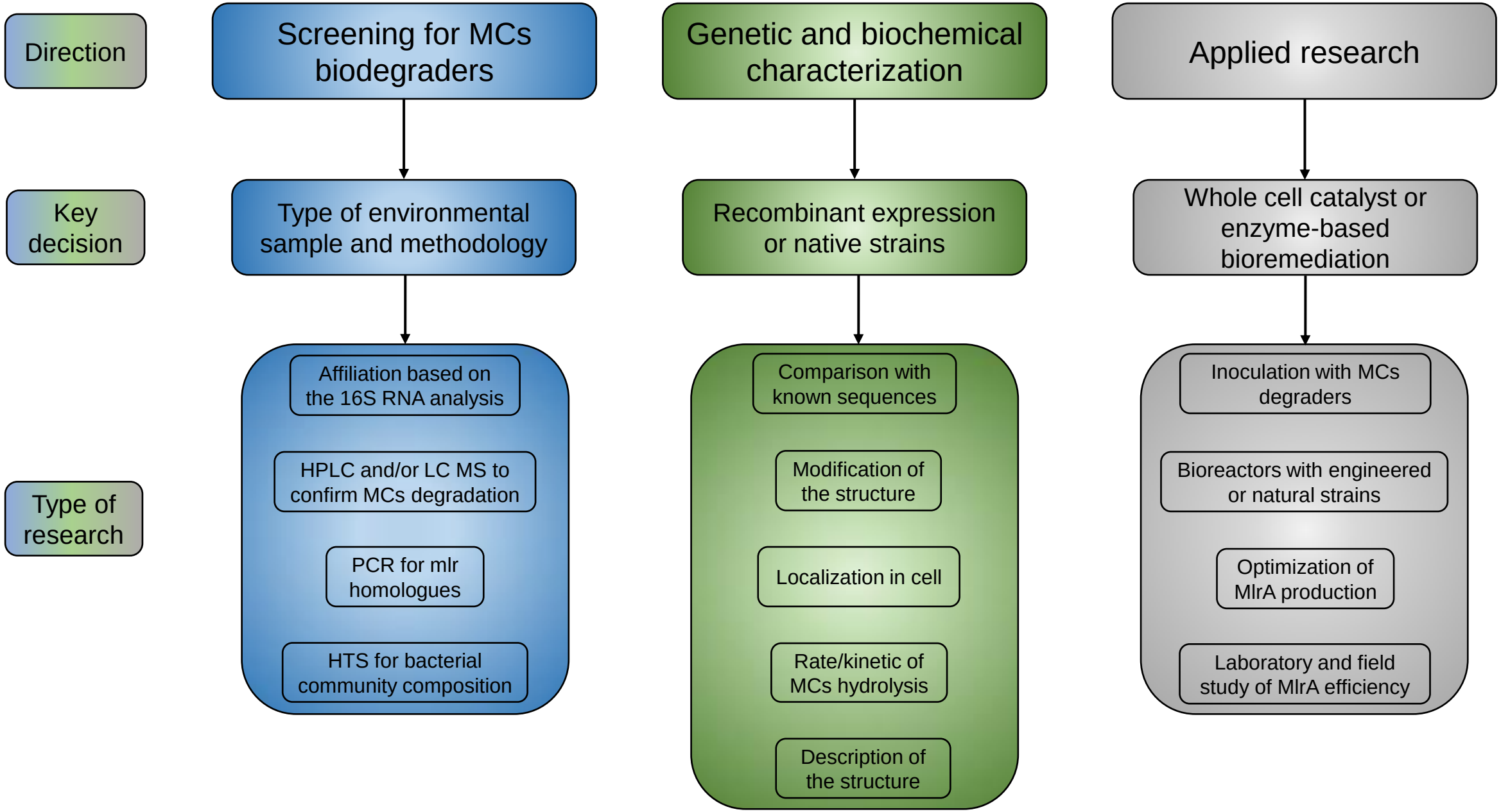
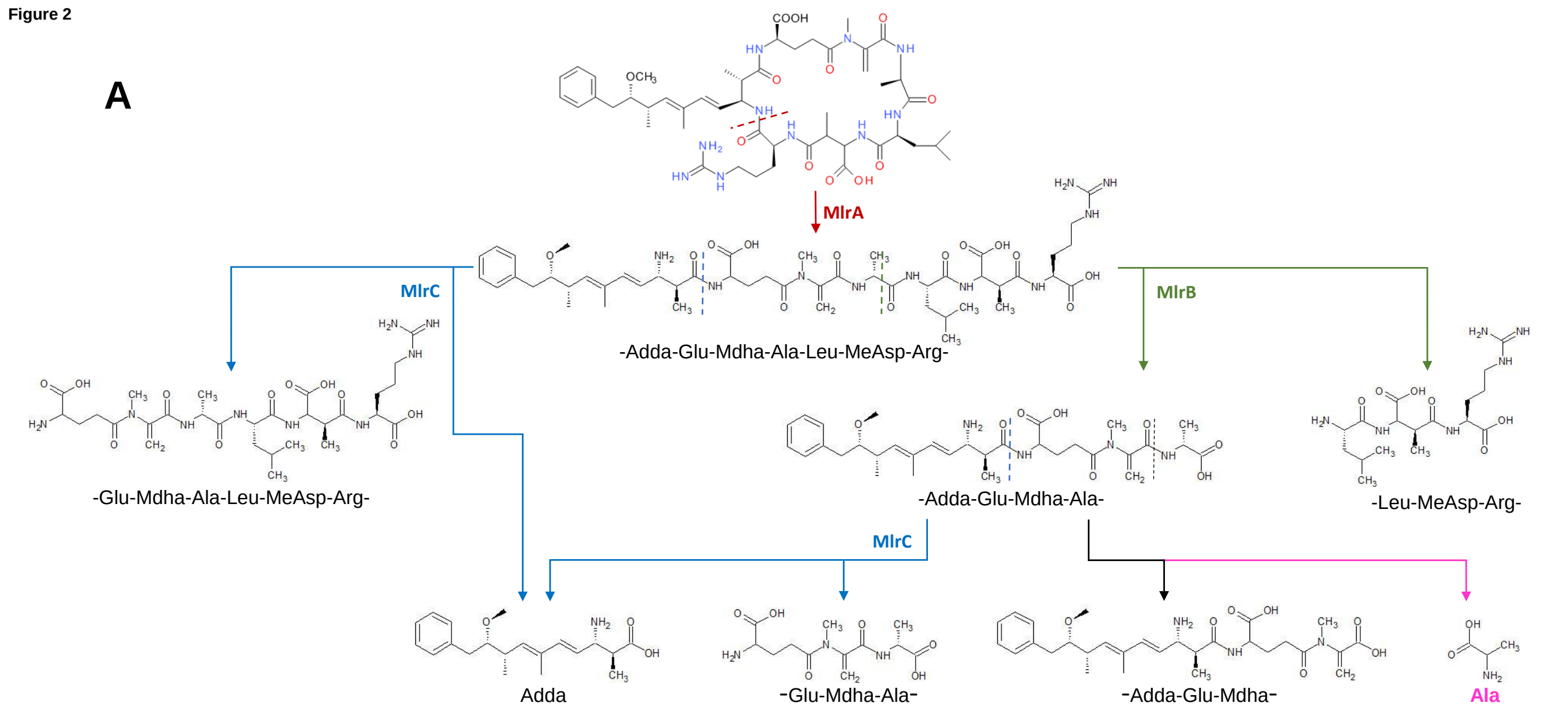
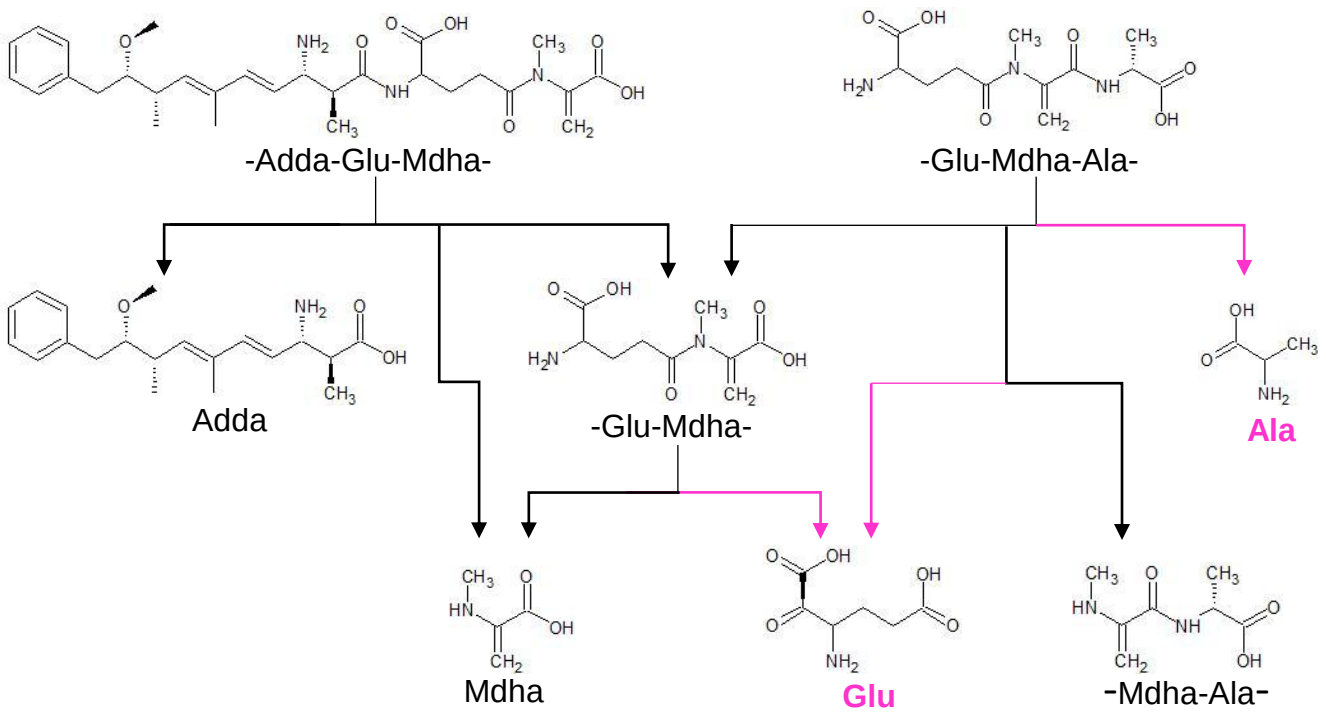


Figure 1. Major directions of the previous and current research on *mlrA*-dependent MCs biodegradation. Blue colors reflect field research ('Screening for MCs biodegraders'), green colors involve basic laboratory study ('Genetic and biochemical characterization'), with gray colors describing biotechnological approaches ('Applied research').

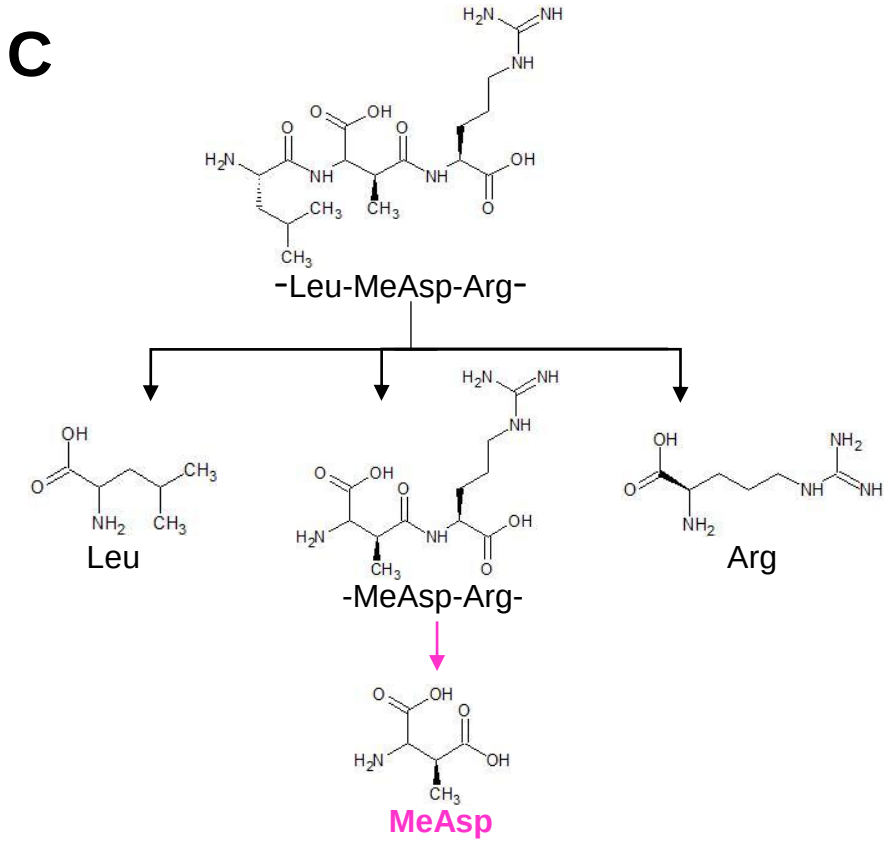
Figure 2


A
MlrA
MlrC
MlrB
-Adda-Glu-Mdha-Ala-Leu-MeAsp-Arg-
-Glu-Mdha-Ala-Leu-MeAsp-Arg-
-Adda-Glu-Mdha-Ala-
-Leu-MeAsp-Arg-
MlrC
Adda
-Glu-Mdha-Ala-
-Adda-Glu-Mdha-
Ala

B



C



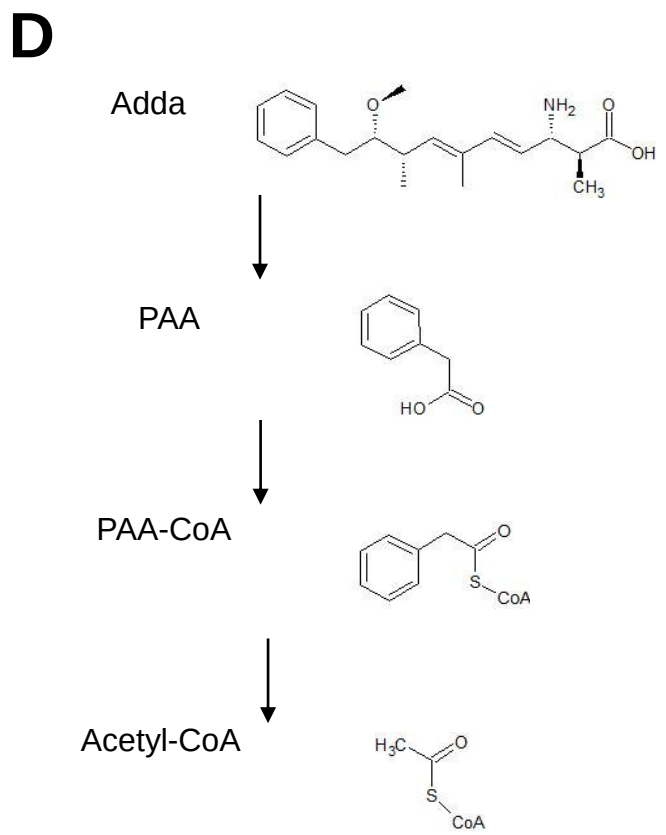


Figure 2. Biochemically verified MlrA-dependent pathway for MC-LR degradation (involves all the intermediates detected and the order of their formation, as described in the cited references, as well as three predicted degradation products [Glu, Ala, MeAsp] marked in pink color). Note, that the degradation products of the hexapeptide have never been determined. (A) Degradation to tripeptides, (B-C) Tripeptides degradation, and (D) Adda degradation.

Figure 3

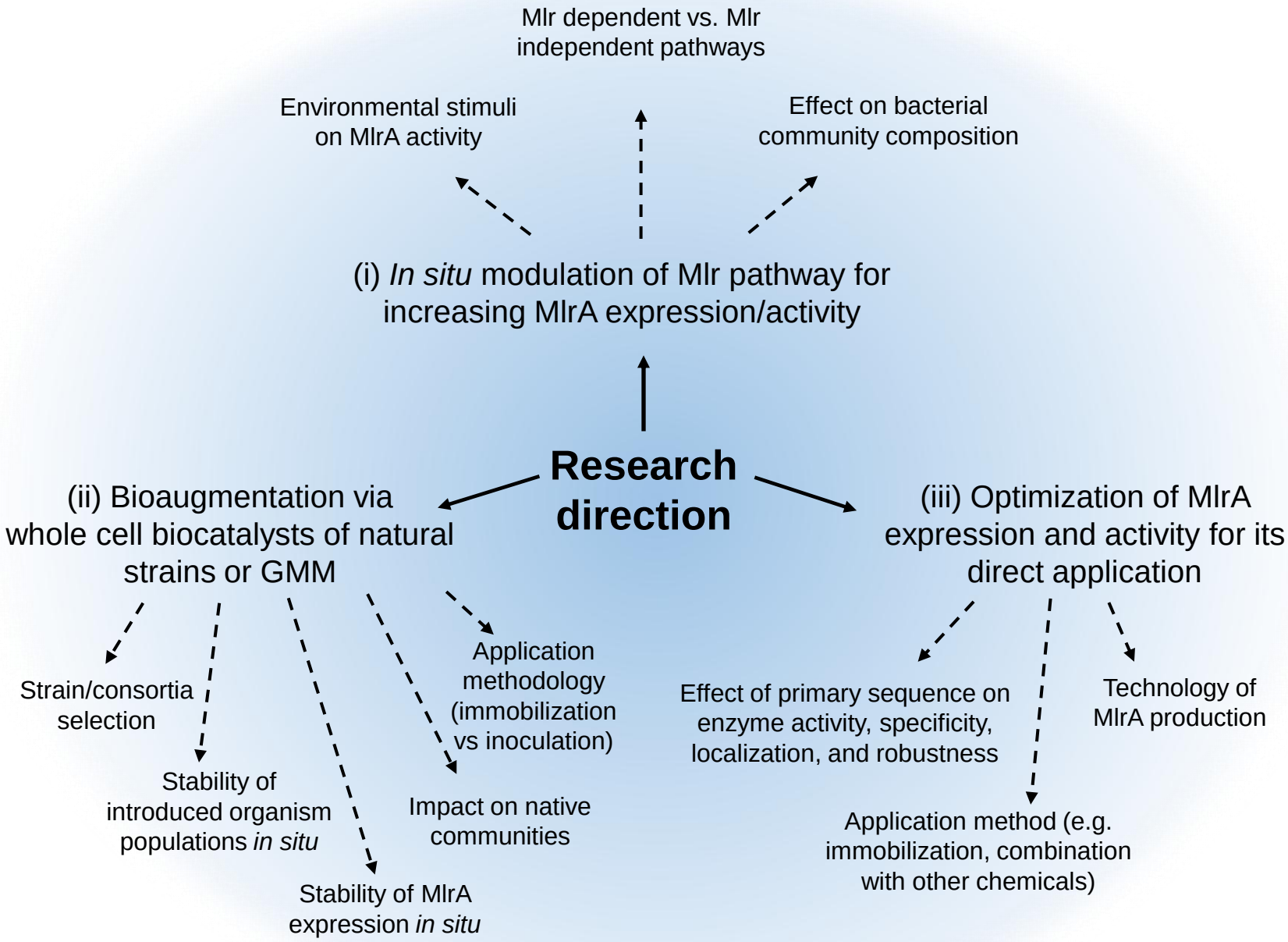


Figure 3. Overview of applied MlrA research directions for technological development. GMM = Genetically modified microorganism.