MICROCYSTIN DEGRADATION IN *Sphingopyxis* sp. C-1

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SUMMARY: The microcystin-degrading gene cluster, *mlrA-B-C-D*, plaies an important role in the degradation process of hepatotoxic microcystins for several bacterial species. However after microcystin is degraded to linear-microcystin by MlrA, it is still unknown about where and by what it is metabolited. In order to clarify it, we disrupted the *mlrB* gene and *mlrC* gene in chromosome of microcystin-degrading bacteria, *Sphingopyxis* sp. C-1. The cells disrupted *mlrB* gene and *mlrC* gene accumulated of microcystin-degradation product, linear-microcystin and tetrapeptide, respectively, whereas the cell free extracts of Δ*mlrB* cells detected Adda and Δ*mlrC* cells accumulated tetrapeptide. Moreover, topology analysis of MlrB using the ß-lactamase gene fusion method insisted MlrB is the peripheral protein binding the inner-membrane. These results insist that MlrB degrades the linear microcystin in the periplasmic space and MlrC degrades tetrapeptide in cytoplasm. Thus, in intact cells, MlrC cannot degrade linear-microcystin as being separated in inner-membrane from linear-microcystin while MlrC is capable of degrading the linear-microcystin in cell-free extract.

Keywords— Microcystin, *Sphingopyxis* sp., Water treatment.

INTRODUCTION

Microcystins are produced in rather high pH by cyanobacteria belonging to the genera *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc*, and are hepatotoxic compounds with cyclic heptapeptides resulting in the death of fishes, birds, many kinds of domestic animals and human [1]. In natural water, biodegradation is main factor of decrease in microcystins [2], which concering gene factors *mlrA*, *mlrB*, *mlrC*, and *mlrD* [3]

In the degradation pathway, we and other groups reported MlrA is the first enzyme to hydrolyze cyclic microcystin LR into a linear intermediate by biochemical and genetic assays. Reacently, some groups reported that *mlrB* would not function from genome analysis *in silico*. But the *E. coli* cells overexpressed *mlrB* or *mlrC* degrades the linear microcystin using the cell free extracts and *mlrB* was verified to function experimentally. But we don't know whether MlrB or MlrC is mainly attributable to degrade the linear microcystin in cells.

This study reports the reason why C-1 cells have both enzymes for degradation of linear-microcystin, and in other words, the reason why *mlrC* doesn't degrade the linear-microcystin in intact cells and the reason that *mlrB* and *mlrC* mainly contribute to degrade the linear-microcystin and the tetrapeptide, respectively.

2. MATERIALS AND METHODS

2.1 Bacterial strains

Spingopyxis sp. C-1 was used as microcystin-degradating bacterium. *Escherichia coli* DH5α was the host in the DNA manipulation.

2.2 Microcystin Degradation Activity.

Degradation of microcystin and the degradation product were assayed by high-performance liquid chromatography (JASCO 2000 Series; JASCO international Co., Ltd, Japan) using a Sun Fire ™C18 column (3.0 × 150 mm; particle size, 5.0 μm; Waters, Milford, MA, USA) at 40ºC.

2.2 Gene modification and DNA techniques.

Deletions of chromosomal genes were conducted by recombination [4, 5]. To obtain *blaM* gene inserted *mlrB* gene to *mlrB* genes, EZ-Tn5 <*blaM/R6Kγori*> transposon (Epicentre) was used. Other molecular biological techniques were followed by the standard procedures described previously or follows the manufacture's instruction [5].

3. RESULTS AND DISCUSSION

3.1 Disruption of *mlrB* gene in chromosome of C-1 strain

In the degradation pathway, MlrA was shown to be a first enzyme to hydrolyze cyclic microcystin LR into a linear intermediate, a linear microcystin LR. The MlrB protein was speculated to hydrolyze linear microcystin LR into a tetrapeptide, which would later be degraded by MlrC. To clear this presumption, we disrupted the *mlrB* gene in chromosome of C-1 strain yielding C1-Δ*mlrB* and verified whether the linear microcystin LR was degradated in C1-Δ*mlrB*. Disruption of chromosomal *mlrB* gene led to accumulate the linear microcystin LR, MlrA degradation product, whereas C-1 cells degradated the microcystin LR completely for 6.5 h.

3.2 Contribution of MlrC in decomposing microcystin LR

The results obtained in 3.2 indicated that MlrB is necessary for degradation but MlrC would not be likely to

contribute to degrade the linear-microcystin in cells. To conferim this speculation, we disrupted *mlrC* gene in chromosome of wild-type strain and C1-Δ*mlrB* mutant yielding C1-Δ*mlrC* and C1-Δ*mlrB*Δ*mlrC*, respectively, and measured the degradative activities of linear-microcystin and tetrapeptide in cells, with companying degradation of microcystin. In C1-Δ*mlrC* strain, the disruption of *mlrC* gene led to accumulate the tetrapeptide. On one hand, in C1-Δ*mlrB*Δ*mlrC* strain, the linear-microcystin accumulated. Moreover, when tetra-peptide was added into C1-Δ*mlrB* cell suspension as a substrate, tetra-peptide decreased immediately, but not in C1-Δ*mlrB*Δ*mlrC* cells at all. This result indicates that MlrC functions in cells and degrades tetra-peptide in cells. When linear-microcystin was added into C1-Δ*mlrC* suspension, it was degraded and decreased immediately, but not suitably in C1-Δ*mlrB*Δ*mlrC* cells at all.

In the next experiments, to clear that MlrC being in cell is incapable of degrading the linear-microcystin, we measured the degradative activities in preparing the cell free extract of C1-Δ*mlrB* and C1-Δ*mlrB*Δ*mlrC* strains. Unexpectedly, the cell free extract of C1-Δ*mlrB* showed clear degradative activity of linear-microcystin and tetrapeptide, but that of C1-Δ*mlrB*Δ*mlrC* didn't do. This result indicated that MlrC is expressing in cytosol and have functions that degrade the linear-microcystin and tetrapeptide, but only a degradative activity of the linear-microcystin was depressed in intact cells by some kind of reasons. One possibility is that the linear-microcystin is separated from MlrC by inner-membrane because MlrA and MlrB are predicted to have a signal sequence by SOSUISignal program, though MlrC doesn't have clear signal sequence and is located in cytosol.

3.3 Construction and Expression of the Reporter Gene, *mlrB*-BlaM Fusion

To determine where the MlrB functions, we constructed *mlrB-blaM* fusions as described in "materials and method." We obtained total 34 independent fusion genes using the transposon insertional method and PCR method. To ascertain proper expression of the hybrid proteins in the *E. coli* host, we employ SDS-PAGE of the 34 cell free extracts prepared from the cells harboring the plasmids, and the proteins were visualized by the immunoblotting method using an antibody raised against ampicillinase. The result depicted that the hybrid proteins from P23-BlaM through P541-BlaM were lined up in increasing molecular mass as the fusion sites became more distal from the carboxyl-terminal end. The size of all hybrid proteins was consistent with the size predicted from the length of the truncated MlrB plus the size of the reporter protein, BlaM. Next, we determined carbenicillin susceptibility of the cells harboring the *mlrB-blaM* fusion gene to ascertain the localization of the hybrid proteins. Because the strains expressing the MlrB-BlaM hybrid protein in the periplasm are expected to be β-lactam-resistant, whereas the strains carrying the fusion in the cytoplasm will be β-lactam-susceptible. The 34 transformants carrying the *mlrB-blaM* fusion exhibited the MICs of carbenicillin 128 μg/ml to more than 256 μg/ml. The MIC of carbenicillin for the host cell harboring pHSG398 without BlaM fusion appeared to be 4 μg/ml and the site of fusion is located in periplasmic space. Moreover, generally, the gaps between one fusion site to the nearest neighboring sites were consistently less than 21 amino acid residues for the membrane topology analysis because about 20 amino acid residues need to span from outside of membrane to inside. These results clearly suggested that the entire MlrB polypeptide would be located at the periplasmic space.

Finally, to ensure our hypothesis, we construct the MlrC-BlaM fusion protein, which was fused ß-lactamase to carbonyl terminal of MlrC protein, and measured the degradating activity and susceptibility to carbenicillin in that transformant. As I had expected, the transformants carrying the plasmid coding the MlrC-BlaM hybrid protein exhibited resistant to carbenicillin and those cell-free extracts had the degradating activity of linear-microcystin and tetra-peptide, suggesting that MlrC functions in cytosol.

4. CONCLUSION

This study revealed the degradation pathway of microcystins in the cell that MlrA firstly hydrolyzed cyclic microcystin LR into a linear intermediate, and MlrB degraded the linear microcystin in the periplasmic space, and then MlrC degraded tetrapeptide in cytoplasm.

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