Short Communication

Lipoproteins Binding Malachite Green to Slow the Decolorization of Malachite Green in *Pseudomonas* sp. JT-1

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Lipoproteins of a malachite green (MG)-decolorizing bacterium *Pseudomonas* sp. JT-1 could bind MG to form green MG-Lipoproteins complexes, which prevented the decolorization of MG by triphenylmethane reductase.

Key words: lipoproteins, malachite green, complexes, decolorization

Malachite green (MG), a chemical primarily designed to be a dye, is used widely to treat parasites, fungal infections and bacterial infections in the aquaculture industry (1, 22). MG is known to be highly toxic to certain freshwater fish and mutagenic and carcinogenic to humans (4, 5, 12, 18). Consequently, most countries have declared MG a hazardous material and prohibited its use in aquaculture. However, it is still used in some areas because of its relatively low cost, ready availability and efficacy (22).

Since MG is a contaminant and potential human health hazard, there is concern about its fate in aquatic and terrestrial ecosystems (3, 15). Studies on the biodegradation of MG have focused primarily on decolorization via reduction reactions (2, 14, 16, 17, 20). In recent years, different bacteria capable of the decolorization of MG have been reported (7, 8, 10, 13, 19). Cultures of *Clostridium perfringens*, *Aeromonas hydrophila* DN322, *Citrobacter* sp. KCTC 18061P, *Pseudomonas* sp. MDB-1, and *Lactobacillus acidophilus* have been shown to convert MG to leuco-malachite green (LMG) (7, 8, 13, 19), which is less toxic to both mammalian and bacterial cells due to its insolubility in water (1). The biochemical mechanism underlying the decolorization has been elucidated in some bacteria. A decolorizing enzyme, triphenylmethane reductase (TMR), was purified from *Citrobacter* sp. KCTC 18061P and characterized (9). The *tmr* gene has also been cloned from *Aeromonas hydrophila* DN322, *Pseudomonas* sp. MDB-1 and an IncP-1 β plasmid pGNB1 from the bacterial community of an activated sludge compartment (13, 19, 21).

Previously, a bacterial strain, JT-1, capable of converting of MG to LMG was isolated and identified as a *Pseudomonas* sp. based on its biochemical characteristics and 16S rRNA gene sequence (GenBank accession number HQ116523). The *tmr* gene (HQ116525) was also amplified from this strain using primers P1 (5'-ATG TCA ATT GCG GTT ACA GGT-3') and P2 (5'-TTA CAT TTT CAG GGC TTG TTT-3'). In the decolorization process, an interesting

phenomenon attracted our attention. After a long (72 h) incubation with MG, little dye was found in the liquid medium; however, a small amount was always detected in the cells. Disruption of these cells and centrifugation yielded colorless supernatant and green insoluble fraction. After extracting the insoluble fraction using organic solvents (as acetonitrile), we detected a small amount of MG. It seemed that this MG was bound in the insoluble fraction.

In order to investigate what bound MG, we separated the components of the insoluble fraction as described by George and Falkinham (6, 24). After the removal of complex lipids, carbohydrates, some soluble proteins, a lipoprotein mixture with MG-binding activity was obtained. The binding reaction was performed by adding lipoproteins (1.0 mg L⁻¹, final concentration) to a reaction mixture containing 10 μ M of MG in 10 mM Tris-HCl buffer (pH 7.5) with 0.1% SDS at 30°C. After 1 min, we separated lipoproteins from this assay solution by ultrafiltration with a Centricon-YM10 filter. The lipoproteins dyed green were retained and the filtrate was colorless. These results indicated that lipoproteins and MG formed MG-lipoproteins complexes, which suggested that the complexes were another form of MG in the micro-environment. Although further purification of lipoproteins from this mixture was successful, many fractions with only a single protein band showed activity to bind MG. So we deduced that a variety of lipoproteins had the binding activity. In order to truly characterize the binding of MG to lipoproteins, we used the lipoprotein mixture in this study.

For experiments *in vitro*, we first cloned the *tmr* gene from JT-1 into the plasmid pUC119 and expressed a His-tagged TMR in *Escherichia coli* BL21 (DE3) as described previously (9). Then the conversion of MG in MG-Lipoprotein complexes was evaluated by using purified His-tagged TMR. The pre-reaction was initiated by adding the lipoprotein mixture (3.0 mg L⁻¹, final concentration) to 10 mL of a reaction mixture containing excessive MG in 10 mM Tris-HCl buffer (pH 7.5) with 0.1% SDS at 30°C for 2 min. The assay solution was subjected to ultrafiltration; then the MG-Lipoprotein complexes were washed twice with 10 mM Tris-HCl buffer (pH 7.5) containing 0.1% SDS and dissolved

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in 40 mL of it to form solution A. Solution A was analyzed by high performance liquid chromatography/mass spectrometry (HPLC/MS) as described (7) and the result showed that the concentration of MG was approximately 20 μM . Conversion I was initiated by the addition of the purified His-tagged TMR protein (0.8 mg L^{-1} , final concentration) with 0.05 mM NADH in solution A at 30°C and stopped by adding two volumes of acetonitrile. Conversion II was performed by adding an equal amount of purified TMR with 20 μM MG and 0.05 mM NADH at 30°C in the same buffer. Samples containing the MG-Lipoprotein complexes and free MG molecules with NADH were run simultaneously under the experimental conditions as control I and II, respectively. All samples were analyzed by HPLC/MS. The results are shown in Fig. 1A. Free MG molecules were converted by TMR rapidly in the first four minutes and hardly detectable from the reaction mixture of conversion II after ten minutes. However, the MG-Lipoprotein complexes were converted

much slower than the free molecules. After ten minutes, 2.8 μM MG could be still detected from the reaction mixture of conversion I. Obviously the complexes to some extent slowed the conversion of MG by TMR. There were two possible causes: (I) Binding to lipoproteins increased the steric hinderance in MG, which decreased the catalytic activity of TMR. (II) Only free MG molecules could be converted by TMR. In solution, lipoproteins, free MG molecules and the complexes maintained a dynamic equilibrium, which could be upset by the conversion of free MG molecules by TMR. Some MG molecules dissociated gradually from the complex and so a new equilibrium would be established, which caused delayed degradation of MG by TMR. No matter what the reason, lipoproteins had a protective effect on MG. Another protein, bovine serum albumin (BSA), has been reported to form a complex with MG (25). In this study, we also investigated the conversion of MG in the MG-BSA complex by His-tagged TMR and compared with that in MG-Lipoprotein complexes. The preparation of complexes and establishment of a reaction system were performed as described above. HPLC/MS showed that MG in the MG-BSA complex was converted much faster than that in MG-Lipoproteins (Fig. 1B). Compared with the control with free MG molecules as a substrate, the conversion of MG in the MG-BSA complex slowed only slightly. These results demonstrated that the protective effect of MG-BSA was apparently weaker than that of MG-Lipoproteins.

Given that lipoproteins play the role of binding MG in JT-1 cells, their over-expression will lead to more binding of MG *in vivo*. In gram-negative bacteria, lipoprotein precursors were synthesized in the cytoplasm and re-directed to the cell wall, where they underwent a second acyl modification by lipoprotein N-acyltransferase (Lnt) to produce triacylated lipoproteins (23). *In vivo*, we cloned the *lnt* gene (GenBank accession number ACB01879) from *E. coli* K-12 substr. DH10B and over-expressed it in JT-1 to produce more lipoproteins. The primers were as follows; primer P₁ adding a *Xho* I site (5'-CCG CTC GAG ATG GCT TTT GCC TCA TTA ATT GAA-3') and primer P₂ adding a *Hind*III site (5'-CCC AAG CTT TTA TTT ACG TCG CTG ACG CAG ACT-3'). The gene was specifically amplified using these primers, *Pyrobest* DNA polymerase (Takara, Ohtsu, Japan), and genomic DNA of *E. coli* DH10B as a template. After digestion with *Xho*I and *Hind*III, the fragment was cloned into the multiple cloning site of the broad-host-range vector pBBR1MCS-2 (11), yielding pBBR1MCS-2A. pBBR1MCS-2A was then transformed into JT-1 by electroporation. The transformant E1 was selected from LB medium containing 50 $\mu\text{g mL}^{-1}$ of kanamycin. Precultures of JT-1 and E1 grown overnight in LB were transferred respectively at 2% (v/v) to fresh M9 medium containing 2% (w/v) yeast extracts and 300 μM of MG, then incubated at 30°C on a rotary shaker. Aliquots (1.0 mL) were taken out periodically and centrifuged. The precipitated cells, after being washed with sterile water, were extracted with 1.0 mL of acetonitrile. The supernatant and the extract were analyzed by HPLC/MS. Although the conversion of MG in the two supernatants showed no significant difference, E1 cells had stronger MG-binding activity than JT-1 cells (Fig. 2). The residual MG in E1 cells reached

Fig. 1. Conversion of MG by TMR. A, Conversion of MG in the absence and presence of lipoproteins. Conversion I (Δ), conversion of MG in MG-Lipoprotein complexes by TMR; Conversion II ($\square$), conversion of free MG molecules by TMR; Control I ($\bullet$), incubation of MG-Lipoprotein complexes without TMR under the same experimental conditions; Control II ($\blacklozenge$), incubation of free MG molecules without TMR under the same experimental conditions; B, Conversion of MG in the presence of PM1 or BSA. Δ , conversion of MG in MG-Lipoprotein complexes by TMR; $\diamond$, conversion of MG in the MG-BSA complex by TMR; $\square$, conversion of free MG molecules by TMR; $\bullet$, incubation of MG-Lipoproteins without TMR under the same experimental conditions; $\blacklozenge$, incubation of MG-BSA without TMR under the same experimental conditions. The error bars indicate standard deviations.

Fig. 2. Conversion of MG in JT-1 and E1 cells. Δ , Residual MG in supernatant of JT-1 cells; $\square$, residual MG in supernatant of E1 cells; $\blacktriangle$, residual MG in JT-1 cells; $\times$, residual MG in E1 cells. The error bars indicate standard deviations.

a maximum value ($7.46 \mu\text{M}$) in 30 h, which was approximately 2.2 times higher than that in JT-1 cells. After 30 h, the amount of MG was decreased in both strains. However, the decrease in the fixed MG occurred much slower in E1 than in JT-1 cells. The amount of MG in E1 cells was $6.43 \mu\text{M}$ at 72 h, 4 times that in JT-1 cells. These results demonstrated that over-expression of the *Int* gene caused JT-1 to bind more MG *in vivo* and the bound MG was difficult to convert, which also suggested that lipoproteins served a binding function.

In summary, in the bacterial strain JT-1, lipoproteins bound with MG to form complexes, which slowed the conversion of MG by TMR. Our findings provide direct evidence of one biochemical mechanism regarding the persistence of MG in an environment containing TMR. Further studies will help us to better understand the environmental behavior of MG.

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