Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2011;20:220–227
DOI: 10.1159/000330669

Published online: August 25, 2011

Decolorizing Activity of Malachite Green and Its Mechanisms Involved in Dye Biodegradation by *Achromobacter xylosoxidans* MG1

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Key Words
Achromobacter xylosoxidans · Malachite green · Biodegradation · *tmr* gene · P450

Abstract
An *Achromobacter xylosoxidans* MG1 strain isolated from the effluent treatment plant of a textile and dyeing factory from Yunnan Province in China was found capable of decolorizing the malachite green dye at a high efficacy. Strain MG1 reduced 86% malachite green at the concentration of 2,000 mg/l within 1 h, representing a greater ability for decolorizing and a higher tolerance of this compound than all previously reported bacteria. Color removal was optimal at pH 6 and 38°C. Further experimental evidences demonstrated that both cytoplasmic and extracellular biodegradation contributed to the decolorization of malachite green. Nested PCR was employed to identify the candidate genes responsible for malachite green decolorization, and we identified a cytoplasmic triphenylmethane reductase gene with 100% amino acid similarity to the corresponding gene in *Citrobacter* sp. strain. In contrast to our expectation, the addition of metyrapone had little effect on the cytoplasmic biodegradation, suggesting that cytochrome P450 was not involved in the high-performance reduction. The extracellular biodegradation was likely attributable to the secretion of extracellular proteases and some heat-resistant compounds.

Introduction

Malachite green, N-methylated diaminotriphenylmethane, has been extensively used in the textile industry for dyeing nylon, wool, silk, leather and cotton. It is also the most efficacious antifungal agent in the fish farming industry due to its relatively low cost, ready availability, and high efficacy against fish microbial pathogens. However, though one of the most common dyes, malachite green is highly toxic to mammalian cells, and is prohibited by the US Food and Drug Administration [Cha et al., 2001]. To decontaminate wastewater, several physicochemical methods have been developed to eliminate the colored effluents containing malachite green [Cooper, 1993]. Unfortunately, most current methods are expensive and produce large amounts of sludge. Therefore, there is a significant interest in developing microbial biodegradation agents to treat such effluents as an alternative candidate [An et al., 2002].

Biological decolorization of malachite green has been studied using a variety of bacteria. *Bacillus cereus* DC11, *Aeromonas hydrophila* DN322, *Pseudomonas otitidis* W/L3, *Mycobacteria*, *Citrobacter* strain KCTC 18061P, *My-*

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cobacteria and a strain from *Kurthia* sp. have shown capable of decomposing malachite green [An et al., 2002; Jones and Falkinham, 2003; Ren et al., 2006; Sani and Banerjee, 1999; Wu et al., 2009]. Furthermore, the molecular mechanisms underlying the biodegradation of triphenylmethane dyes have been partially elucidated. HPLC analysis to the intestinal bacteria *Clostridium perfringens* and *Lactobacillus acidophilus* cultures incubated with malachite green showed that malachite green could be converted into leucomalachite green [Henderson et al., 1997]. Jang et al. [2005] purified the enzyme triphenylmethane reductase (TMR) from *Citrobacter* strain KCTC 18061P, and showed that this enzyme is efficient at decolorizing malachite green to the colorless leucomalachite green. Additionally, metyrapone inhibited the decolorization of malachite green by *Mycobacteria* sp. such as *Mycobacterium chelonae* and *Mycobacterium avium*, implying the involvement of cytochrome P450 in the decolorization reaction [Jones and Falkinham, 2003].

A. xylosoxidans is a Gram-negative, oxidase-positive, non-glucose-fermenting rod that is widely distributed in the environment. It is usually considered nonpathogenic but can cause serious infections, such as bacteremia, meningitis and peritonitis, in hospitalized patients [Sepkowitz et al., 1987; Wellinghausen et al., 2006; Weitkamp et al., 2000]. It is also an endophytic bacterium in plants. It was revealed that the inoculation of *A. xylosoxidans* F3B to *Arabidopsis thaliana* resulted in a significant increase in root length and fresh weight [Ho et al., 2009]. Similarly, it also has the abilities to degrade p-nitrophenol, MTBE, bisphenol A [Eixarch and Constanti, 2010; Wan et al., 2007; Zhang et al., 2007]. However, there is still no report describing the decolorization of malachite green by *A. xylosoxidans*. In this paper, we report the isolation and characterization of a new strain of *A. xylosoxidans* MG1, and demonstrate that this strain is capable of decolorizing malachite green at a high efficiency. We also investigated its potential biodegradation mechanisms.

Results

Identification and Acclimation of a Dye-Decolorizing Bacterium

A strain with a relatively high ability to decolorize malachite green (78 ± 3% decolorizing efficiency at 40 mg/l dye concentration within 24 h) was isolated from the effluent treatment plant of a textile and dyeing factory in Yunnan Province, China. Microscopic observations identified it as a Gram-negative rod. When the sequence of its 16S rRNA gene was compared with those in GenBank, it was shown to share 98% similarity with the known sequences from *A. xylosoxidans*, suggesting that this new isolate was a member of *A. xylosoxidans*. Our strain was submitted to China Center for Type Culture Collection (CCTCC) and was stored as No. M203045.

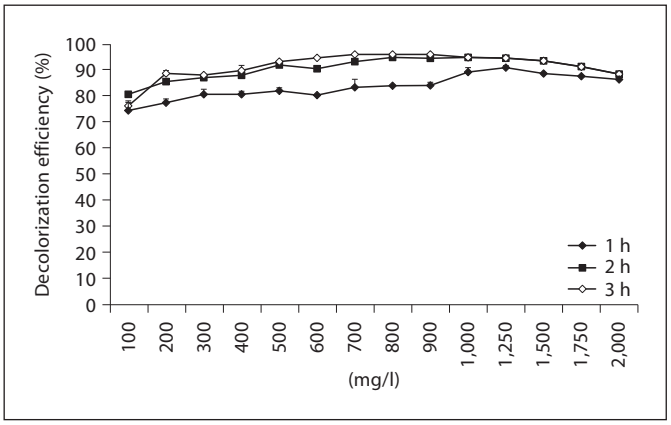

Fig. 1. Decolorization efficiencies of the strain *A. xylosoxidans* MG1 at different concentration of malachite green. The x values represented different concentrations of malachite green, and the y values represented the decolorization efficiencies.

Before further study, we performed five acclimations that increased the strain's malachite green decolorization efficiency significantly. The results of decolorization assay after acclimation indicated that this strain had a higher decolorizing ability even at high concentrations of malachite green than other bacteria reported previously. For example, at the concentration of 2,000 mg/l dye, about 86 ± 1% of color was removed in 1 h by the acclimated strain. However, the decolorization efficiency did not vary tremendously for dye concentrations from 40 to 2,000 mg/l (86.2 and 88.5%, respectively). On the contrary, it seemed that higher concentrations of malachite green could be more effective at inducing the decolorizing activity (fig. 1). Furthermore, strain MG1 was also able to decolorize crystal violet, although the decolorization efficiency was much lower than that against malachite green (data not shown). This difference between decolorization efficiencies of the two compounds could be due to their different chemical structures, among which malachite green has two dimethyl groups in two side chains whereas crystal violet has three dimethyl groups in three side chains [Rajesh and Uttam, 1999].

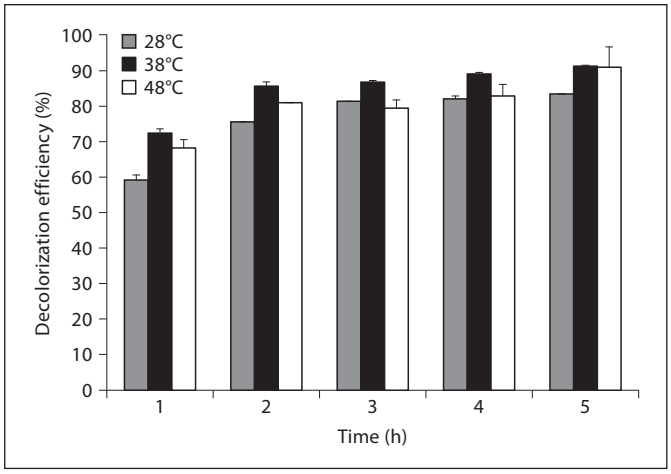

Fig. 2. Effects of temperature on the decolorization efficiency of *A. xylosoxidans* MG1. The x values represented different times for decolorization, and the y values represented the decolorization efficiencies.

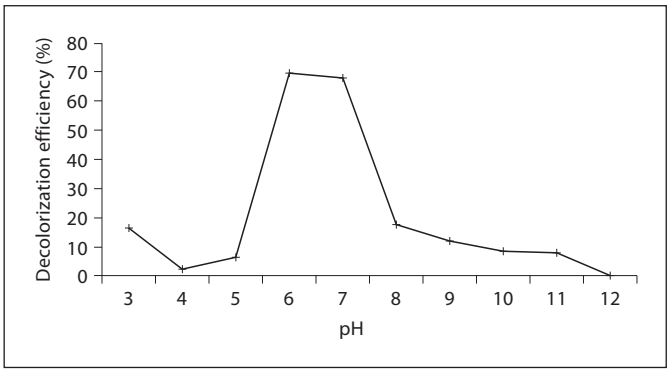

Fig. 3. Effects of pH on the decolorization efficiency of *A. xylosoxidans* MG1. The x values were the tested pHs, and the y values were the decolorization efficiencies.

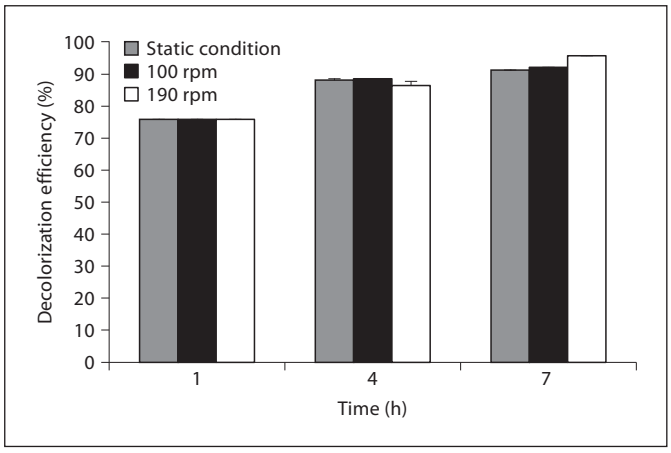

Fig. 4. Effects of rotation speed on the decolorization efficiency of *A. xylosoxidans* MG1. The x values represented different time for decolorization, and the y values represented the decolorization efficiencies.

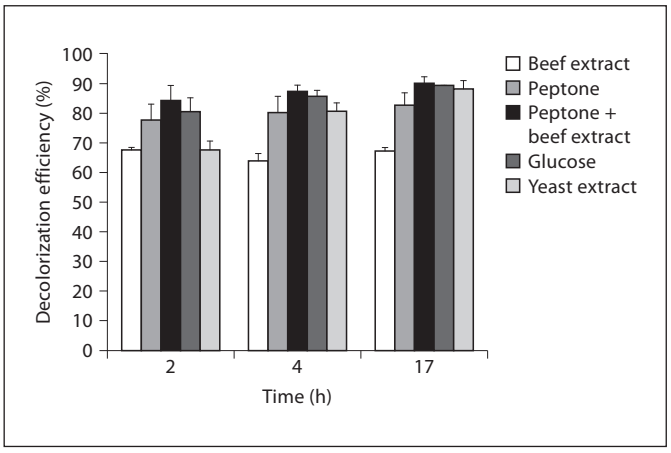

Fig. 5. Effects of carbon source on the decolorization efficiency of *A. xylosoxidans* MG1. The x values represented different times for decolorization, and the y values represented the decolorization efficiencies.

Effects on the Decolorization Activities

The different temperature treatments (e.g. 28, 38 and 48°C) showed little influence on the decolorization activities of strain MG1 ($p > 0.05$) (fig. 2). In contrast, different pH treatments showed significant differences in decolorization activities, likely through affecting the stability of malachite green (fig. 3). The optimum decolorization activity was observed at pH 6.0 and 38°C in the LB decolorization medium. Similar decolorizing efficacies were observed when the strain was grown under conditions of constant shaking, certain microaerophilic environments, and static incubation. The results thus suggest that the decolorization reaction was largely independent of the concentration of molecular oxygen ($p > 0.05$) (fig. 4). A further experiment was designed to confirm the impact of oxygen on the decolorization efficiency, in which a layer of mineral oil was covered above the medium, and we obtained the same results.

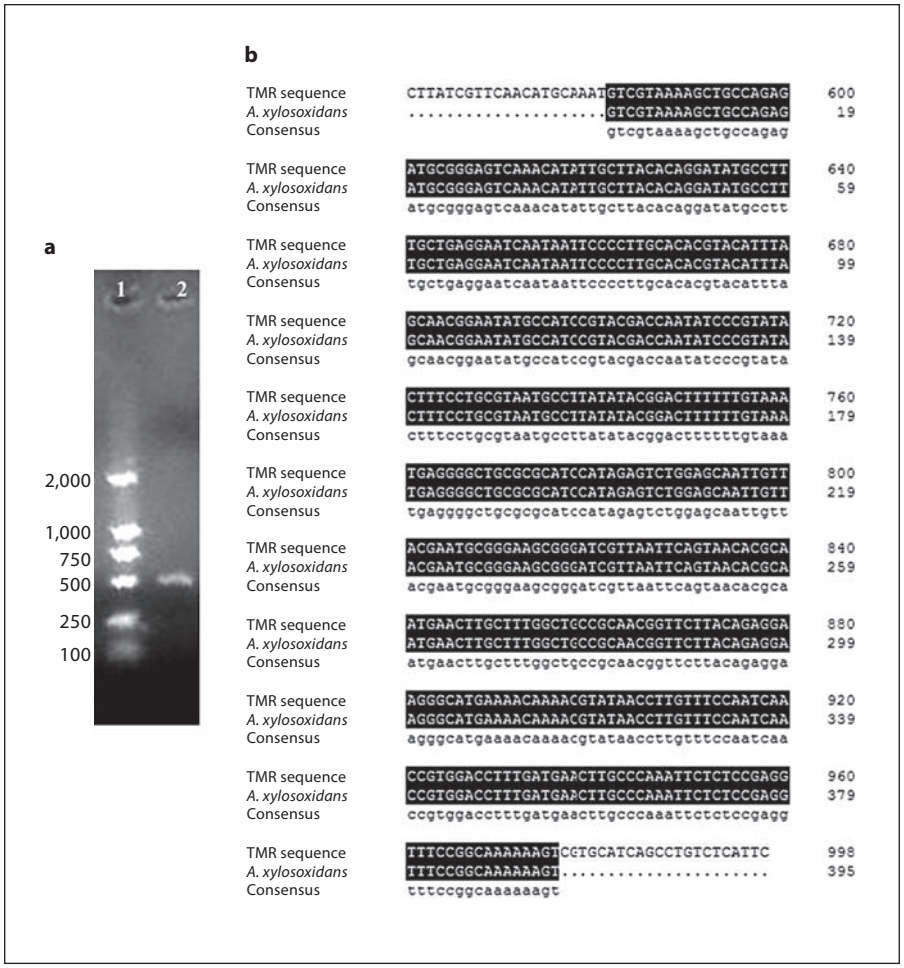

Fig. 6. The gene *tmr* in *A. xylosoxidans* MG1. **a** The result of nested PCR and the amplified fragment was 510 bp. Lane 1 represented the DNA marker of DL 2000; lane 2 was the target PCR products. **b** The alignment of *tmr* genes from *A. xylosoxidans* MG1and *Citrobacter* sp.

Several different carbon sources were chosen to test their effects on dye decolorization efficiency by *A. xylosoxidans* MG1 (fig. 5). We found that strain MGI could degrade malachite green in M9 medium containing all the tested carbon sources, among which the highest decolorization efficiency occurred in a mixed carbon source environment with peptone and beef extract. Using yeast extract as the carbon source, the initial decolorization efficacy was found relatively low but was increased significantly later on. Altogether, the effects on decolorization efficiencies from different carbon sources had no significant differences in ANOVA test.

Cloning of Gene tmr

Nested PCR was undertaken to isolate the gene tmr, which has been shown capable of reducing malachite green in *Citrobacter* sp. strain KCTC 18061P (fig. 6b) [Jang et al., 2005]. After two sequential PCR with strain MG1 genomic DNA as template, a specific amplicon of about 500 bp was obtained as we had expected (fig. 6a). This fragment was sequenced and then compared to those related genes in GenBank. The *tmr* gene in MG1 shared 100% amino acid sequence similarity with tmr in *Citrobacter* sp. strain KCTC 18061P (fig. 6b) [Jang et al., 2005]. Our result suggests that *tmr* in *A. xylosoxidans* MG1 likely performs a similar function as that in *Citrobacter* sp. strain KCTC 18061P, contributing to the decolorization activity of malachite green.

Inhibition of Metyrapone on Decolorization

As the inhibitor of a cytochrome P450, metyrapone has been shown capable of inhibiting the decolorization of malachite green by pathogenic *Mycobacteria* such as *M. chelonae* and *M. avium* [Guerra-Lopez et al., 2007; Jones and Falkinham, 2003]. Thus, we measured the effect 10 mM metyrapone on the decolorization reaction of

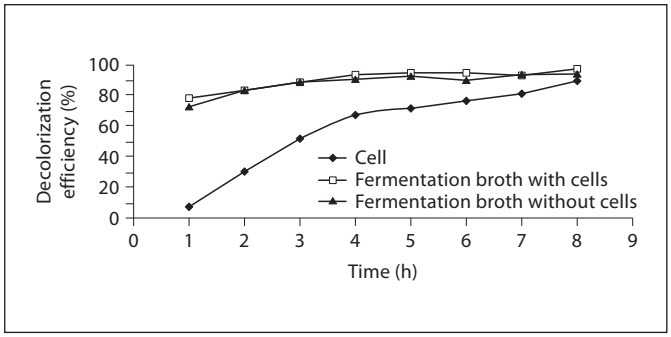

Fig. 7. Comparisons of the decolorization abilities by the bacterial cells, the supernatant without cells, and the fermentation broth.

malachite green by *A. xylosoxidans* MG1 to determine whether P450 participated in this reaction. Our result showed that the decoloration efficiency from extracellular fraction was about 36.7 ± 0.2% after adding metyrapone comparing to 34.0 ± 0.9% in the negative control; at the same time, the decoloration efficiency from membrane fraction was about 65.4 ± 1.7% while the negative control had the decoloration efficiency of 64.1 ± 0.6%. Conclusively, this result demonstrated that the addition of metyrapone had no inhibition effects on the decolorization efficiency of *A. xylosoxidans* MG1, and thus mono-oxygenase cytochrome P450 should be unlikely involved in decoloration of malachite green in *A. xylosoxidans* MG1.

Extracellular Decolorizing Activity

In addition to dye biodegradation resulting from the cytoplasmic TMR, the extracellular culture filtrate without bacterial cells also showed relatively high decolorization efficiency for malachite green. It was also observed that the extracellular activity of dye biodegradation was faster than that containing only bacterial cells inoculated into fresh culture at the beginning stage of decolorization. But when the bacterial cells had grown for 8 h after fresh inoculation, the activity to remove color returned to normal (fig. 7). Furthermore, after heating to denature the extracellular enzymes, the fermentation filtrate still possessed partially decoloration activity though it could not remove the color completely. Taken together, these results suggested that strain MG1 contained extracellular enzyme(s) as well as some other heat resistant extracellular compounds responsible for biodegradation of malachite green.

Discussion

Many investigations have been performed in the area of dye biodegradation. Deng et al. [2008] reported the degradation of malachite green by *B. cereus* DC11 and its decoloration efficiency was 96 ± 4% with 55 μM (about 20 mg/l) malachite green within 3 h. It was also reported that the decoloration activity of *A. hydrophila* DN322 was >90% in 10 h when the concentration of malachite green was up to 50 mg/l [Ren et al., 2006]. A strain of *Citrobacter* sp. reduced malachite green by more than 80% in 1 h at the concentration of 100 μM (about 36.5 mg/l) [Guerra-Lopez et al., 2007], and *P. otitidis* W/L3 removed 95% of malachite green within 12 h at 500 μM (about 182 mg/l) dye concentration [Wu et al., 2009]. Additionally, it was also found the several mycobacterial strains could tolerate a very high concentration of malachite green, up to 120 μg/ml (about 43.8 mg/l). Our strain, identified as a member of *A. xylosoxidans* by the combination of morphologic and phylogenetic analysis, has shown the highest decolorizing and tolerance abilities when compared to other bacteria reported previously. The strain analyzed here could decolorize about 86 ± 1% of malachite green in 1 h even at the concentration of 2,000 mg/l.

Microbiological decolorization of dyes may take one of two strategies: either adsorption by the microbial biomass or biodegradation of the dyes by the cells [Zhou and Zimmermann, 1993]. Dye adsorption may be evident from inspection of the bacterial growth: those adsorbing dyes will be deeply colored, whereas those causing degradation will remain colorless [An et al., 2002]. *A. xylosoxidans* strain MG1 in our current study likely employs both mechanisms. We observed that the cells and pellets were colored slightly at the initial stage of dye biodegradation. However, as the time went on, the color in the supernatant outside bacterial cells disappeared completely depending on microbial biodegradation.

In this study, we also found that both the intracellular and extracellular components contributed to the decolorizing activities against malachite green. Our nested PCR identified the *tmr* gene in this strain. This gene encodes a previously known cytoplasmic enzyme that plays an important role in the reduction of triphenylmethane dyes in *Citrobacter* sp. Strain KCTC18061P. A subsequent study demonstrated that this gene exists in a plasmid pGNB1, a broad-host range plasmid, in a bacterial community of a wastewater treatment plant. Here, the *tmr* gene from the promiscuous plasmid pGNB1 was flanked by IS1071, and conferred decolorization and resistance to

triphenylmethane dyes such as crystal violet, malachite green and basic fuchsin [Schluter et al., 2007]. In strain *A. xylosoxidans* MG1, the gene *tmr* was located on the chromosome, similar to that of *Citrobacter* sp. These results suggest that broad-host range plasmids such as pGNB1 in natural environment might be involved in transferring genes among different hosts.

Cytochrome P450 is a protein superfamily containing hundreds of closely related hemeproteins. It belongs to the mono-oxygenases that can add one oxygen atom to the substrate. Cytochrome P450 is also one of the most common catalysts in bacteria for decomposing certain types of organic compounds [Hannemann et al., 2007]. The roles of cytochrome P450 in dye decolorization have also been documented in the fungus *Cunninghamella elegans* ATCC36112, the bacteria *M. chelonae* and *M. avium* based on the evidence that their decolorizing activities could be inhibited by metyrapone [Cha et al., 2001; Jones and Falkinham, 2003]. However, in our strain, the addition of metyrapone had no effect on its decolorization activity, suggesting cytochrome P450 was unlikely involved in the dye reduction of our strain.

Extracellular biodegradation of malachite green has also been investigated in fungi. It is revealed that a few species of fungi employ the non-specific degradation enzymes to decolorize various dyes. Lignin peroxidase from *Phanerochaete chrysosporium* could decolorize crystal violet [Bumpus and Brock, 1988]. Hardin et al. [2000] also showed that the peroxidase enzymes LiP and MnP in the activities of decolorization. Laccase from the extracellular fraction of *Cyathus bulleri* decolorized triphenylmethane dyes [Vasdev et al., 1995]. So far, a few fungal genes encoding lignin peroxidase and laccase with the activity of removing dyes have been cloned and characterized [Cullen, 1997; Gold and Alic, 1993]. Whether similar extracellular enzymes are responsible for the extracellular biodegradation of malachite green in *A. xylosoxidans* needs further elucidation.

In conclusion, our strain of *A. xylosoxidans* MG1, which was isolated from an effluent treatment plant, could decolorize high concentrations of malachite green efficiently. Furthermore, the decolorization of malachite green involved both biodegradation and adsorption. Because multiple pathways for malachite green degradation are likely present in *A. xylosoxidans* MG1, this strain could have significant potential in solving the dye pollution problem.

Experimental Procedures

Chemicals and Bacterial Strains

All the dyes used in this study, including crystal violet and malachite green, are of analytical grade and purchased from Kermel Co. All other chemicals used were also of analytical grade. The bacterial strains capable of decolorizing malachite green were screened from textile and dye-stuff effluent in Yunnan Province, China.

Isolation and Identification of Bacterial Strains for Decolorizing Dye

The screening of the bacterial strains for dye decolorization was carried out on agar plates containing (g/l): yeast extract 5, NaCl 10, tryptone 10, agar 20, and malachite green 40 mg/l. Acclimation to improve the decolorizing activity was carried out in liquid medium containing (g/l): KH_2PO_4 0.5, K_2HPO_4 1.5, NaCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, NH_4Cl 1.0 and a certain amount of malachite green increased from 50 to 100 mg. Every concentration of the acclimation lasted 5 days.

After purification by successive single colony isolation on Luria-Bertani (LB) plate, a strain with the most significant activity was selected. Bacterial identification was based on a combination of phenotypic, biochemical properties and 16S rDNA sequences amplified by PCR using two universal primers [Yoon et al., 1998]. The sequences of the amplified 16S rDNA gene was analyzed and compared to those related sequences in GenBank.

Assay of Malachite Green Decolorization

Decolorization of dyes was determined by monitoring the decrease in absorbance at the maximum wavelength of malachite green which is at 617 nm. An UV-Visible scanning spectrophotometer (Beckman DU640) was used for absorbance measurement and recording of UV visible absorption spectra. The activity was determined by the percentage decolorization, a modified method described previously [Yatome et al., 1993]. After cells were removed by the centrifugation at 12,000 rpm for 2 min, the supernatant samples were used for absorbance measurement. All assays were performed in at least three duplicates and the data shown here were the averages.

Decolorization rate (%) = 100 (A – B)/A, where A = initial absorbance and B = observed absorbance.

Effects on the Decolorization of Malachite Green

To determine the factors that influenced the effects of decolorization, cells were aerobically cultured in 250 ml flasks containing 90 ml LB medium at 38°C with shaking at 190 rpm overnight till cell density reached at OD_{600} 0.50–0.60.

In the assay to determine the effects of temperature on decolorization, the cultures with 10^9 cells/ml were incubated at various temperatures (28, 38 and 48°C) in rotary shakers when a moderate concentration 40 mg/l of malachite green was used. To test the effects of pH, the same cultures were respectively adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 by using HCl or NaOH [Harrelson and Mason, 1982]. The impact of oxygen content on the decolorization was also measured with shaking condition (100 and 190 rpm) or without shaking (static condition). The methods for collecting the supernatant samples as well as the decolorization assays were the same as those described above.

Additionally, a variety of carbon sources were chosen to test their effects on dye decolorization. The decolorizing medium was M9 synthetic medium with different carbon sources containing: 12.8 g/l $Na_2HPO_4{\cdot}7H_2O$, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1.0 g/l NH_4Cl added 40 mg/l malachite green. The different carbon sources included 5.0 g/l yeast extracts, 10 g/l peptone, 3 g/l beef extract, 10 g/l glucose and the mixture of 3 g/l beef extract and 5 g/l peptone for fish [Harrelson and Mason, 1982]. After cells were pre-cultured as the method above, the supernatant were removed by the centrifugation at 8,000 rpm for 10 min. Then the bacterial cells were inoculated into the fresh decolorizing medium with different carbon sources. At each sampling time, the supernatants were collected for decolorization assay.

Inhibition of Metyrapone on Decolorization of Dyes

The membrane and soluble fractions were prepared as described with minor modification [George and Falkinham, 1989]. Fermentation broth was centrifuged at 8,500 rpm for 15 min at 4°C. The pellet was washed in 50 mM sodium phosphate buffer (pH 7.0) containing 1.0 mM EDTA. After the cells were disrupted by sonication in an ice bath (on 5 s, off 7 s, time 15 min, 48% amplitude), $MgCl_2$ was added to a final concentration of 2.0 mM. The suspension was cleared by centrifugation at 12,000 rpm for 1 min at 4°C to yield the crude extract fraction in the supernatant. This supernatant fraction was centrifuged at 12,000 rpm for 30 min at 4°C to separate the cytoplasmic membrane (pellet) and soluble (supernatant) fractions. The pellet was washed with 50 mM sodium phosphate buffer (pH 7.0) with 1.0 mM EDTA and 2.0 mM $MgCl_2$. The suspension, pellet, as well as the mixture of them were all added 100 mM metyrapone solution to a final concentration of 10 mM, while the negative control used the same volume of ddH_2O instead of the metyrapone. Before the adding malachite green with the final concentration of 10 mg/l, they were incubated for 10 min at 37°C [Jones and Falkinham, 2003].The result was monitored by a UV-visible scanning spectrophotometer as above.

Extracellular Decolorizing Activity

Cells were cultured in a 250-ml flask containing 100 ml LB medium at 38°C with shaking at 190 rpm overnight. When the absorbance at 600 nm reached about 0.5, the bacterial cells were removed by the centrifugation at 8,000 rpm for 10 min and subsequently at 10,000 rpm for 20 min. Malachite green were then added to the extracellular culture to the final concentration of 40 mg/l. After incubation for a specific time, the reaction mixtures were used as samples for decolorization assay. As controls, the blank LB and fermentation broth without centrifugation were used.

Candidate Genes Involved in Malachite Green Decolorization

A cytoplasmic TMR has been revealed to be responsible for decolorizing triphenylmethane dyes in *Citrobacter* sp. strain KCTC 18061P [Jang et al., 2005]. To identify if this gene was present in the malachite green decolorization strain in *A. xylosoxidans* MG1, two pairs of primers for nested PCR were designed as follows. P1 (F) 5′ AGTCAAATCATTGCCATCGTG 3′; P1 (R) 5′ GGTTTCCTTCAGAGGGGTC 3′; P2 (F) 5′ GCTCTTTATCTCAGGTCCGC 3′; P2 (R) 5′ ACACCAGCGTTTACGAGGA 3′. The amplification system of PCR in 50 μl included the template 1 μl, primer1 1 μl, primer2 1 μl, 10 × Taq buffer 5 μl, dNTP mixture (2.5 mM) 4 μl, Taq (2.5 U/μl) 1 μl, and ddH_2O 37 μl. PCR amplification was performed under the following conditions: 35 cycles of 94°C 30 s, 56°C 30 s, 72°C 1 min. The amplicon of nested PCR was sequenced and compared to those of related genes in GenBank.

Statistical Analysis

All the data are expressed as mean values (SD). Comparisons among multiple groups were made with a one-way analysis of variance (ANOVA) followed by Dunnet t test. $p = 0.05$ was used for statistical significance.

Acknowledgement

We are grateful to Dr. Jianping Xu of McMaster University in Canada for his comments and revisions. This work was supported by the National Basic Research Program of China (Program 863, grant No. 2007AA021303), and the Department of Science and Technology of Yunnan Province, China (grant No. 2006PY01-27).

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