Biodegradation (2009) 20:769–776
DOI 10.1007/s10532-009-9265-z

ORIGINAL PAPER

Isolation of a malachite green-degrading *Pseudomonas* sp. MDB-1 strain and cloning of the *tmr2* gene

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Received: 25 November 2008 / Accepted: 29 April 2009 / Published online: 26 May 2009

Abstract The release of malachite green, a commonly used triphenylmethane dye, into the environment is causing increasing concern due to its toxicity, mutagenicity, and carcinogenicity. A bacterial strain that could degrade malachite green was isolated from the water of an aquatic hatchery. It was identified as a *Pseudomonas* sp. based on the morphological, physiological, and biochemical characteristics, as well as the analysis of 16S rRNA gene sequence and designated as MDB-1. This strain was capable of degrading both malachite green and leucomalachite green, as well as other triphenylmethane dyes including Crystal Violet and Basic Fuchsin. The gene *tmr2*, encoding the triphenylmethane reductase from MDB-1, was cloned, sequenced and effectively expressed in *E. coli*. These results highlight the potential of this bacterium for the bioremediation of aquatic environments contaminated by malachite green.

Keywords Malachite green · Triphenylmethane dyes · Biodegradation · *Pseudomonas* sp. · *tmr2* gene

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Introduction

Malachite green (4-[(4-dimethylaminophenyl)-phenyl-methyl]-*N*, *N*-dimethyl-aniline, MG), a triphenylmethane dye, has been widely used since the 1930s in the aquaculture industry as an anti-fungal and anti-parasitic agent (Hoffman and Meyer 1974; Alderman 1985; Daneshvar et al. 2007). It is also extensively used in the food, health, textile, and other industries (Srivastava et al. 2004). However, the physical and chemical properties of MG make it difficult to remove from aqueous solutions. If effluent containing MG is discharged into natural aquatic systems, it can affect the aquatic life and, in fish, can cause detrimental effects in the liver, gills, kidneys, intestines and gonads (Daneshvar et al. 2007). Malachite green can also be transformed to leucomalachite green (LMG), which accumulates in the tissues of fish and other aquatic food species and then enters the human food chain. MG poses significant health risks for consumers, exerting effects on the immune system and reproductive system, in addition to having genotoxic and carcinogenic potential (Srivastava et al. 2004). Although the use of MG has been banned in fisheries in the United States, Japan, China, the European

Union, and many other countries, it is still being used illegally as a practically irreplaceable disinfectant, due to its low cost, ready availability, and antifungal effectiveness (Schnick 1988; European Communities 2006; Sudova et al. 2007). Random samplings of fish from markets in the UK, Netherlands, and China indicate the continuing use of MG in the aquaculture industry (Veterinary 1996; Bergwerff and Scherpenisse 2003; Lin et al. 2006). Consequently, methods for the removal of MG from water are of significant scientific and public health interest.

Biological degradation, being inexpensive and eco-friendly, is considered a valuable removal method for many toxic pollutants (Utkarsha et al. 2008). Several microorganisms, including a number of bacteria, yeast, and fungi, have been investigated for their ability to biodecolorize triphenylmethane dyes (Wamik et al. 1988; Sani and Banerjee 1999; Cha et al. 2001; An et al. 2002; Liu et al. 2004; Jadhav and Govindwar 2006; Ren et al. 2006b; Utkarsha et al. 2008). Biochemical studies of the decolorization process indicate that laccase, peroxidase, and lignin peroxidase from fungi and triphenylmethane reductase (TMR) from bacteria are involved in the enzymatic decolorization of dyes (Tekere et al. 2001; Shin and Kim 1998; Jang et al. 2005).

The TMR from *Citrobacter* sp. strain KCTC 18061P has been purified and biochemically characterized, and the gene (*tmr*) encoding this enzyme has been cloned (Jang et al. 2005). This same gene has also been found in *Aeromonas hydrophila* (Ren et al. 2006b). In the genus *Pseudomonas*, *P. aeruginosa* has been shown to decolorize MG (Lin et al. 2004), but the genes associated with the decolorization or degradation of MG have not yet been investigated. In this paper, we report on the isolation and characterization of a new bacterial strain MDB-1 from the genus *Pseudomonas* that shows a potent degradation capability for both MG and LMG. Results on functional gene cloning, sequencing, and over-expression of the *tmr2* gene from this species are also presented.

Materials and methods

Medium

The MG mineral salt medium (MGM, pH 7.0) contained NaCl (0.5 g l^{-1}), K_2HPO_4 (1.5 g l^{-1}), KH_2PO_4 (0.5 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.2 g l^{-1}), and MG (final concentration 10 mg l^{-1}, added just before use unless otherwise stated). Luria–Bertani (Sambrook et al. 1989) medium (LB, pH 7.0) contained tryptone (10.0 g l^{-1}), yeast extract (5.0 g l^{-1}), and NaCl (10 g l^{-1}).

Chemicals and analytical methods

Malachite green (malachite green hydrochloride, MG) and leucomalachite green (LMG), were purchased from Sigma–Aldrich (Steinheim, Germany). Crystal Violet, Basic Fuchsin, Methylene Blue, Coomassie Brilliant Blue, Alizarin Red S and Orange II were purchased from Shanghai Sangon Co, Ltd, China. All other chemicals used were of analytical grade.

Malachite green degradation was determined by the decrease in absorbance at 622 nm using an UV–Visible scanning spectrophotometer (PC-2401). For the other dyes, the MG in the MGM was substituted by equal amounts of Methylene Blue, Crystal Violet, Coomassie Brilliant Blue, Basic Fuchsin, Alizarin Red S, or Orange II. The decrease in absorbance of each dye was determined with its characteristic wavelength using an UV–Visible scanning spectrophotometer (PC-2401) (Table 1). The amount of degradation was calculated from the difference between the initial and final absorbance values and expressed as a percentage of the original concentration.

The degradation of LMG was determined by HPLC using the following procedure: A 3 ml aliquot

Table 1 Degradation of different dyes by *Pseudomonas* sp. MDB-1

Substrate	Absorption maximum (nm)	Degradation[a] within 48 h incubation (%) ± SE
Crystal violet (triphenylmethane)	584	95.61 ± 0.6
Basic fuchsin (triphenylmethane)	542	91.36 ± 0.8
Coomassie brilliant blue (non-azo)	554	23.85 ± 1.6
Methylene blue (thiazine)	662	11.30 ± 1.4
Alizarin red S (anthraquinone)	508	3.32 ± 1.2
Orange II (diazo)	484	2.07 ± 1.2

[a] Values shown are the means of triplicate cultures

SE Standard error

sample of culture media was extracted with an equal volume of dichloromethane. The extract was dried over anhydrous Na_2SO_4 and then evaporated under reduced pressure using a centrifugal evaporator at room temperature. The organic residue was redissolved in 3 ml acetonitrile, and 20 µl of the solution was injected onto a reverse-phase high-performance liquid chromatography column for HPLC analysis (RP-HPLC, 600 Controller, Rheodyne 7725i Manual injector and 2487 Dual λ Absorbance Detector; Waters Co, Milford, MA). The column (internal diameter, 4.6 mm; length, 25 cm) was filled with Kromasil 100-5C18. The mobile phase was acetonitrile: water (80:20, V: V), the flow rate was 1.0 ml min^{-1}, and the UV detector was set at 266 nm. The retention time of LMG was 6.7 min. Concentration of LMG in the samples was determined by comparison of peak areas to that generated by injection of an authentic LMG standard.

Enrichment and isolation of the MG-degrading *Pseudomonas* strain

An enrichment culture method (Ou and Sharma 1989) was used to isolate the MG-degrading *Pseudomonas* strain. The water sample was collected from an aquatic hatchery that routinely uses MG as a disinfectant. About 10 ml of the sample was added to an Erlenmeyer flask (250 ml) containing 100 ml MGM and incubated at 30°C on a rotary shaker at 150 rpm for about 7 days. Five milliliters of this culture was then subcultured five times at 7 day intervals. The enriched culture was serially diluted and spread onto a MGM agar plate. After 3 days, colonies were selected according to their MG degrading capabilities. One strain, designated MDB-1, was selected for further investigation.

Identification of the strain

The identification of MDB-1 was carried out according to Bergey's manual of determinative bacteriology (Holt et al. 1994) and its 16S rRNA gene sequence. Genomic DNA was extracted by high-salt-concentration precipitation (Miller et al. 1988). The 16S rRNA gene was amplified by PCR (Lane 1991) and ligated with pMD18-T (TaKaRa Biotechnology, Dalian, China), then transformed into *E. coli* DH5α. The sequence was determined by an automatic sequencer (Applied Biosystem, model 3730). Alignment of the 16S rRNA gene sequence was performed by ClustalX 1.8.3 (Thompson et al. 1997) with default settings. Phylogenesis was analyzed by MEGA version 3.0 Software. Distances were calculated using the Kimura two-parameter distance model. Unrooted trees were built by the Neighbor Joining method. The dataset was bootstrapped 1,000 times (Weisburg et al. 1991).

MG degradation by MDB-1

After culturing MDB-1 in 50 ml of LB overnight at 30°C with shaking at 180 rpm, cells were harvested by centrifugation at 6,000×g for 5 min, and then washed twice with 25 ml of sterilized 0.5 M phosphate buffer (pH 7.0). The cells were diluted to 10^8 cells ml^{-1} and 1 ml was inoculated into 100 ml MGM and incubated under the same conditions for 48 h. At 6 h intervals, 5.2 ml of the cultures were sampled. Serial dilutions of 0.2 ml aliquots were used for estimation of colony forming units (cfu), while a 5 ml aliquot was centrifuged at 5,000 rpm for 15 min and the supernatant was used to determine the degradation of MG (2 ml) and LMG (3 ml). For controls, samples containing no bacteria and samples containing no MG were run simultaneously with the experimental samples. The former controlled for non-specific MG degradation by components in the medium, while the latter indicated the growth of MDB-1 in MGM lacking MG.

Effects of temperature (15–40°C), initial medium pH (4.0–9.0), and initial MG concentration (5–50 mg l^{-1}) on the degradation of MG were studied. Media without inoculation were used as controls for all of these tests.

Degradation of repeated addition of MG aliquots

To examine the repetitive degradation capability of MDB-1, consecutive cycles of MG degradation were studied by repeated additions of MG (10 mg l^{-1}) in the medium at each cycle.

Preparation of MDB-1 cell free extracts and enzyme assays

Pseudomonas sp. MDB-1 cells were grown in nutrient broth at 30°C for 24 h, then centrifuged at 10,000 rpm for 20 min. These cells were suspended

in sodium phosphate buffer (50 mM, pH 7.4) and sonicated at 4°C. The homogenate was centrifuged and the supernatant was used as a cell free extract (crude enzyme) (Kalme et al. 2006). Triphenylmethane reductase activity in the cell free extract was measured spectrophotometrically as described by Jang et al. (2005). The standard assay system for triphenylmethane reductase consisted of 20 mM sodium phosphate buffer (pH 7.0), 20 μM MG, 0.1 mM NADH, and a suitable amount of the enzyme in a total volume of 1 ml. Each reaction was initiated by the addition of the enzyme, and the initial reaction rate was determined by monitoring the decrease in absorbance at 622 nm in the first 2 min. All enzyme assays were carried out at 30°C. Reference blanks contained all components except the enzyme.

Cloning, sequencing, and expression of the *tmr2* gene in *E. coli*

The triphenylmethane reductase gene, *tmr2*, was cloned from MDB-1 by PCR. The primers were designed according to the *tmr* gene (Jang et al. 2005) as follows: The forward primer contained a *Nde*I restriction site (underlined) and an initiation codon ATG (boldfaced) (5′-CTCAT**ATG**TCAATTGCGGT TACAGGTGCTAC-3′), reverse primer contained a *Xho*I restriction site (underlined) (5′-CACTCGAGT TACATTTTCAGGGCTTGTTTTACGG-3′). Genomic DNA of MDB-1 was used as template; PCR products were purified and cloned into pMD18-T vector (TaKaRa Biotechnology, Dalian Co, Ltd, China), yielding pMD-*tmr2*, which was transformed into competent *E. coli* DH5α cells. The recombinant plasmid in positive transformants was used as the template for direct sequencing of the *tmr2* gene by an automatic sequencer (Applied Biosystems, model 3730). The NdeI/XhoI fragment of pMD-*tmr2* was introduced into the corresponding sites of the pET-29a(+) plasmid, resulting in pET-*tmr2*. *E. coli* BL21(DE3) cells transformed with pET-*tmr2* were grown in LB medium containing kanamycin (50 μg ml^{-1}) at 37°C with a shaking speed of 200 rpm. When the culture turbidity reached 0.6 at 600 nm (about 3 h), protein production was induced by addition of 1 mM isopropyl-1-thio-*β*-D-galactopyranoside (IPTG). The protein extracts prepared from the induced culture at 0 (just before IPTG addition), 1, 2, 3 and 4 h were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.0% SDS–PAGE) according to the method of Laemmli (Laemmli 1970).

Results

Isolation and characterization of strain MDB-1

Strain MDB-1 was selected for its relatively rapid growth and high MG degrading efficiency. It was a Gram-negative, rod-shaped (0.8–1.0 × 1.6–2.0 μm) bacterium with a polar flagellum. Biochemical characteristics were as follows: catalase activity, positive; oxidase activity, positive; nitrate reduction, positive; fermenting of glucose and fructose, positive; hydrolysis of starch, positive; Voges Proskauer test, negative; indole test, negative; liquefaction of gelatin, negative. The 16S rRNA gene sequence of MDB-1 was deposited at GenBank under Accession No. DQ981492. Comparison with available sequences in GenBank showed a high similarity of MDB-1 with species in the genus *Pseudomonas*. A phylogenetic tree based on known representatives of *Pseudomonas* species is presented in Fig. 1. On the basis of the above characters, strain MDB-1 was given a preliminary identification as a *Pseudomonas* sp.

MG degradation by MDB-1

The kinetics of MG degradation and MDB-1 growth were investigated simultaneously (Fig. 2). More than 90% of added MG disappeared within 12 h but no equivalent accumulation of LMG occurred. The degradation of LMG initially was slower than the MG transformation, but after 12 h LMG degradation was more rapid than the MG transformation. MDB-1 cells continued to proliferate in the presence of MG, with an exponential phase of growth occurring between 24 and 36 h. An approximately threefold increase in cell numbers was seen after 48 h cultivation. No growth occurred in cultures lacking MG and uninoculated controls showed no notable degradation of MG. After 48 h, no MG could be detected in the culture, but the levels of LMG continued to decrease beyond the 48 h time point (data not shown). To confirm that the decolorization of MG by MDB-1 was due to degradation and not to adsorption, cell extracts of MDB-1 were tested for their ability to degrade

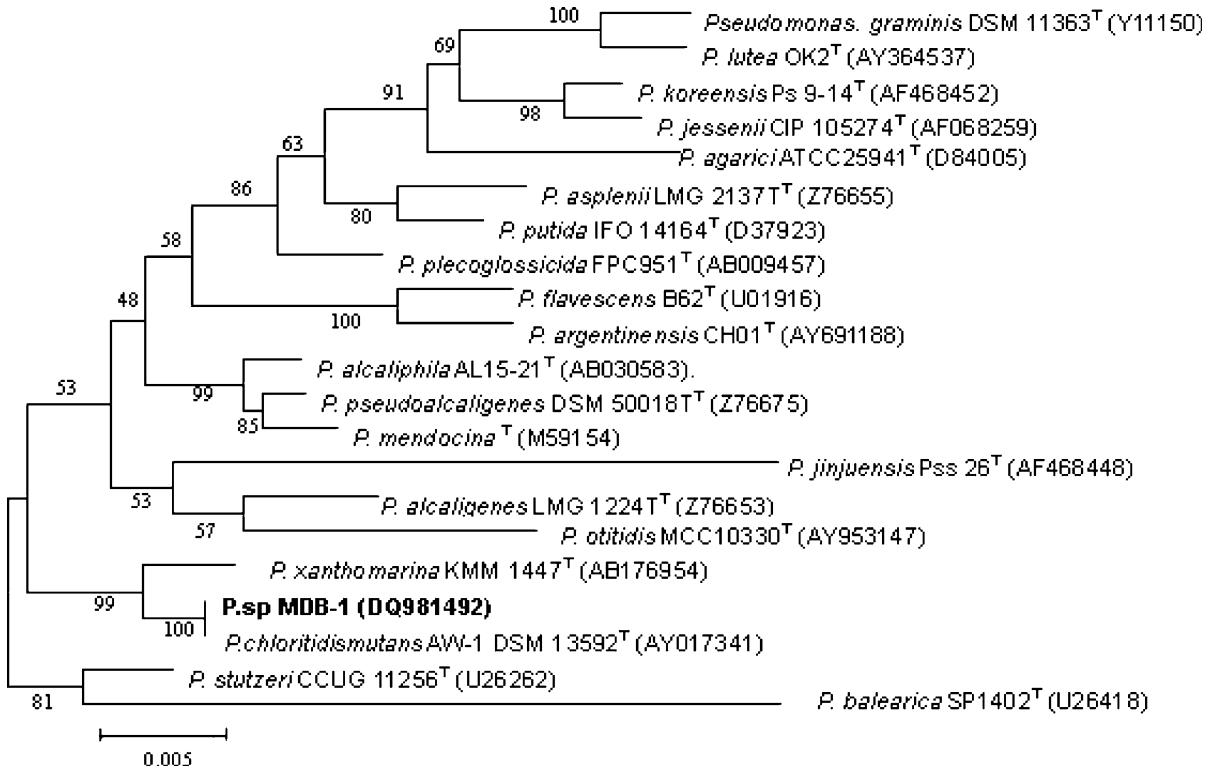

Fig. 1 Dendrogram illustrating the (16S rDNA gene) similarity of the MG-degrading bacterium (*MDB-1*) to strains exhibiting highest sequence similarity (RDP analysis and FASTA)

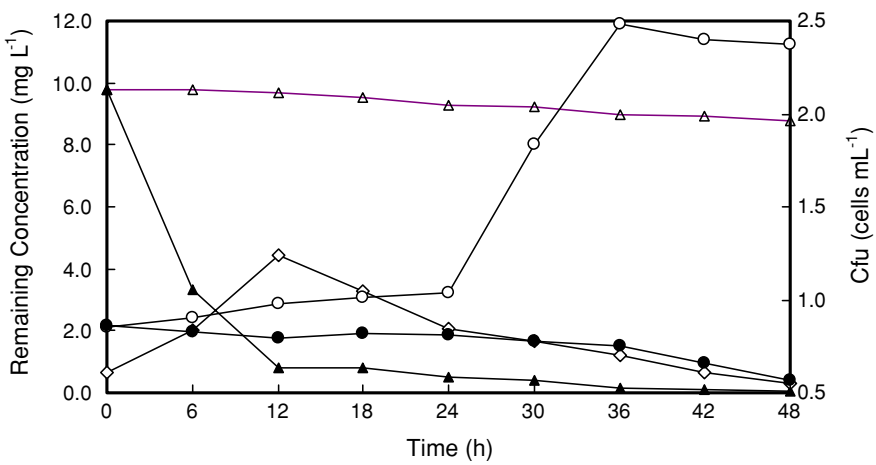

Fig. 2 MG Degradation by MDB-1. △ MG concentrations in uninoculated control; ▲ MG concentrations of inoculated cultures; ○ Cfu of MDB-1 cultures (cells $ml^{-1} \times 10^7$); ● Cfu of MDB-1 in lacking MG control (cells $ml^{-1} \times 10^7$); ◇ LMG concentrations of inoculated cultures. Each point represents the mean of three replicate measurements; standard error of the mean was <5%

MG. A substantial decolorization occurred and triphenylmethane reductase activity and LMG were both detected, which confirmed that the decolorization of MG by MDB-1 was a true enzymatic degradation process (data not shown).

Effects of temperature and initial medium pH on MG degradation by strain MDB-1 were also investigated. The optimal pH and temperature for MG degradation were 8 and 30°C, respectively. The MG degradation of the controls without inoculation was negligible at pH or temperature tested.

The effects of initial concentration of MG degradation are shown in Fig. 3. The amount of degradation varied with initial MG concentration and increased as

the initial MG concentration was raised. In the first 6 h, all of the tested concentrations indicated a fast degradation; the amount of MG decreased 40–50%, then the degradation rate decreased. Initial MG concentration also affected the efficiency of degradation. As the initial concentration of MG increased from 5 to 50 mg l^{-1}, the amount of degraded MG increased from 1 to 84 μg h^{-1} within 48 h.

Degradation of repeated additions of MG

Consecutive cycles of MG degradation were estimated by repeated addition of MG (10 mg l^{-1}) into the medium. After 5 cycles, the degradation efficiency showed no significant decrease (Fig. 4).

Substrate range

Strain MDB-1 could also degrade other dyes, as shown in Table 1. Within 48 h, MDB-1 was able to degrade more than 90% of the Crystal Violet and Basic Fuchsin, indicating that it has significant degradation efficiency for triphenylmethane dyes in general. In contrast, for non-azo and thiazine dyes, it had little degradation efficiency i.e., <30%. No degradation capability was seen for diazo or anthraquinone dyes.

Cloning, sequencing, and expression of *tmr2* gene

The sequence analysis of the *tmr2* gene showed an 864 bp length (GenBank accession No. EF463103), with 99% similarity to the *Citrobacter* sp. MY-5 triphenylmethane reductase gene (*tmr*) (AY756172)

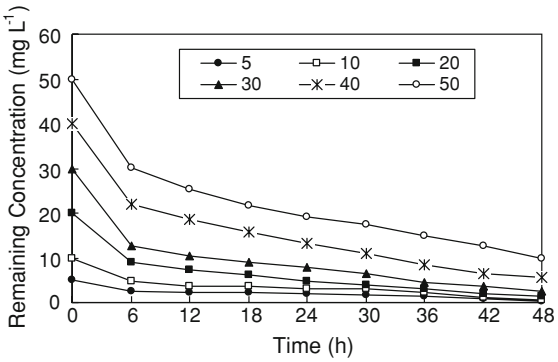

Fig. 3 Effect of initial concentration of MG on degradation of MG. Each point represents the mean of three replicate measurements; standard error of the mean was <5%

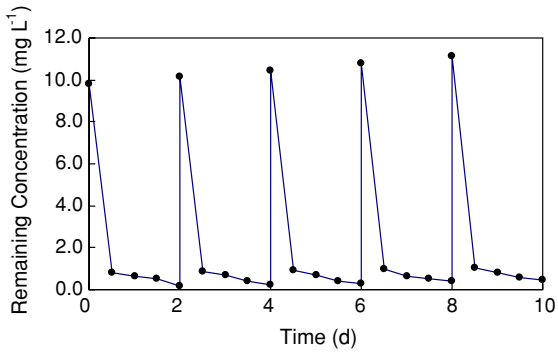

Fig. 4 MG degradation in a batch-fed process. Each point represents the mean of three replicate measurements; standard error of the mean was <5%

and the *Aeromonas hydrophila* subsp. strain DN322 triphenylmethane oxygenase gene (tpmD) (EF010984). The deduced amino acid sequence showed 100% similarity with these two genes.

The recombinant *E. coli* BL21 with plasmid pET-*tmr2* was induced with 1 mM IPTG at 37°C for 3 h, and then plated on MGM agar plates. The recombinants produced clear transparent zones after 12 h of incubation at 37°C. Their ability to degrade MG was further confirmed in liquid MGM culture. Expression of the *tmr2* gene in *E. coli* BL21(DE3) and SDS–PAGE analysis showed strong expression of the *tmr2* gene encoding protein. The molecular mass of the enzyme was about 31 kDa (Fig. 5), which was consistent with the molecular mass of TMR in strain *Citrobacter* sp. (Jang et al. 2005).

Discussion

In the present study, a MG-degrading bacterial strain MDB-1 was isolated and identified as a member of the *Pseudomonas* genus by the analysis of physiological and biochemical characteristics and its 16S rRNA gene sequence. Although some strains of the genus of *Pseudomonas*, including *Pseudomonas pseudomallei* 13NA, *Pseudomonas* spp., *Pseudomonas mendocina* MCM B-402, *Pseudomonas aeruginosa*, *Pseudomonas* sp. and *Pseudomonas putida*, have been reported to decolorize triphenylmethane dyes (Yatome et al. 1981; Sarnaik and Kanekar 1995; Sarnaik and Kanekar 1999; Lin et al. 2004; Oranusi and Ogugbue 2005; Chen et al. 2007), only *Pseudomonas aeruginosa* has been shown to decolorize MG. However, its

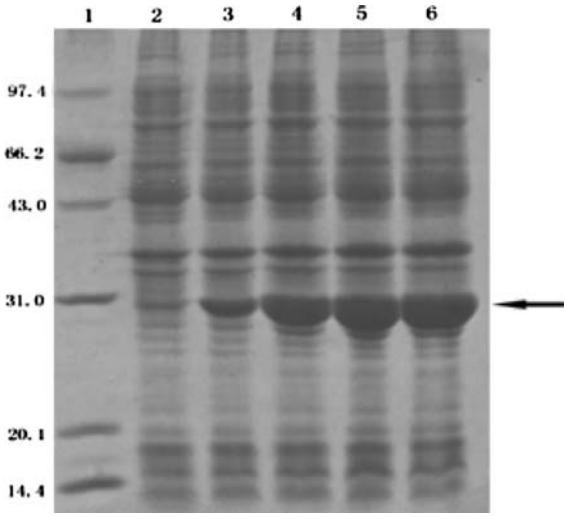

Fig. 5 Expression of the *tmr2* gene in *E. coli*. *Lane 1* molecular mass markers (in kilodaltons); *lane 2* protein extracts prepared from uninduced cultures of *E. coli* BL21(DE3) cells; *lanes 3–6*, protein extracts prepared from cultures of *E. coli* BL21(DE3) cells induced for 1, 2, 3 and 4 h, respectively. Expression of *tmr2* is indicated by an *arrow*

decolorizing mechanism has not yet been elucidated (Lin et al. 2004). To the best of our knowledge, *Pseudomonas* sp. MDB-1 was the first strain from the genus of *Pseudomonas* that has been shown to degrade not only MG but also its metabolite, LMG. Most of the previously reported MG-degrading strains have been isolated from soils, effluent, or sludge samples collected from sites contaminated by textile industry processes, in which the MG concentrations were high. MDB-1 studied here is the first strain isolated from water samples collected from an aquatic hatchery, where MG concentrations were low. This indicates that MG-degrading strains can exist in environments with high as well as low concentrations of MG. The MDB-1 strain was also able to degrade other triphenylmethane dyes, including Crystal Violet and Basic Fuchsin, in addition to MG. Its excellent degrading ability suggests that it has potential applications in the treatment of wastewater from the dye and aquaculture industries.

Triphenylmethane reductase has been shown to be responsible for the decolorization and degradation of MG to LMG in many strains (Henderson et al. 1997; Jadhav and Govindwar 2006; Jang et al. 2005). Jang et al. (2005) purified and characterized the triphenylmethane reductase (TMR) from *Citrobacter* sp. strain KCTC18061P, which catalyzed the NAD(P)H-dependent reduction of triphenylmethane dyes and which had a substrate specificity that was dependent on the chemical structure of the triphenylmethane dyes. The enzyme was a heme-containing reductase with a homodimeric structure and a subunit size of about 31 kDa. The gene *tmr* encoding TMR was first cloned from *Citrobacter* sp. strain KCTC18061P (Jang et al. 2005). Ren et al. (2006a, b) reported the properties of the triphenylmethane dye decolorizing enzyme TpmD from *Aeromonas hydrophila* strain DN322, which was purified to homogeneity and identified as an NADH/NADPH-dependent, heme-containing oxygenase with a molecular of 87 kDa. The encoding gene of TpmD was also cloned and sequenced. In the present study, the gene *tmr2* cloned from the MDB-1 strain was 99% similar to *tmr* from *Citrobacter* sp. strain KCTC18061P and *tpmD* from *Aeromonas hydrophila subsp.* strain DN322. Therefore, this study is the first report of cloning of the *tmr2* gene from the genus *Pseudomonas*. Sequence alignments revealed that this gene is considerably conserved in these strains; however, whether this gene is conserved in other triphenylmethane degrading strains needs further study.

Acknowledgments This work was supported by Agricultural Technology Transfer Programs (grant 2008) and National Program for Basic S and T Platform Construction (2005DKA21201-2).

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