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ORIGINAL PAPER

Biodegradation of leuco derivatives of triphenylmethane dyes by *Sphingomonas* sp. CM9

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Abstract A leuco derivatives of triphenylmethane dyes degrading bacterium, strain CM9, was isolated from an aquafarm field. Based on morphology, physiologic tests, 16S rDNA sequence, and phylogenetic characteristics, it was identified as *Sphingomonas* sp. This strain was capable of degrading leucomalachite green (LMG), leucocrystal violet and leucobasic fuchsin completely. The relationship between bacterium growth and LMG degradation suggested that strain CM9 could use LMG as the sole source of carbon. The most LMG degradation activity of CM9 crude extract was observed at pH 7.0 and at 30°C. Many metal ions had little inhibition effect on the degradation activity of the crude extract. CM9 also showed strong decolorization of triphenylmethane dyes to their leuco derivatives. GC/MS analysis detected two novel metabolic products, methylbenzene and 4-aminophenol, during the LMG degradation by CM9.

Keywords Leuco derivatives of triphenylmethane dyes · Degradation · Decolorization · Metabolic products

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Introduction

Synthetic dyes are extensively used in the textile industries and significant proportion appears in the form of wastewater and is spilled into the environment. A major class of synthetic dyes includes the azo, anthroquinone and triphenylmethane dyes, all of them are generally considered as the xenobiotic compounds, which are very recalcitrant to biodegradation (US Environmental Protection Agency 2005).

Triphenylmethane dyes are aromatic xenobiotic compounds that are used extensively in many industrial processes, such as textile dyeing, paper printing, and food and cosmetic manufacture (Alderman 1985; Schnick 1988). They are known to be highly toxic to mammalian cells and mutagenic and carcinogenic to humans (Case and Pearson 1954; Fernandes et al. 1991; Littlefield et al. 1985; Rao 1995). Based on their potential for adverse human health effects, most countries have nominated triphenylmethane dyes as hazardous material and prohibited the use of them in aquaculture and food industry. However, they are still used in some areas due to their relatively low cost, ready availability and efficacy (Schnick 1988).

Since triphenylmethane dyes occur as contaminants and potential human health hazards, there is concern about the fate of them in aquatic and terrestrial ecosystems (Burchmore and Wilkinson 1993; Nelson and Hites 1980). Studies on the biodegradation of triphenylmethane dyes have focused primarily on the decolorization of them via

reduction reactions (Azmi et al. 1998; Michaels and Lewis 1986; Ollikka et al. 1993; Pointing and Vrijmoed 2000; Sani and Banerjee 1999). Several species of fungi are known to decolorize triphenylmethane dyes by a variety of enzymes as laccase (Casas et al. 2009; Vasdev et al. 1995), peroxidase (Shin and Kim 1998), lignin peroxidase (Bumpus and Brock 1988) or cytochrome P450 monooxygenase (Cha et al. 2001) and the structural genes encoding laccase and lignin peroxidase have been cloned and characterized (Cullen 1997; Gold and Alic 1993). In recent years different bacteria capable of triphenylmethane dyes decolorization have been reported (Ayed et al. 2009; Henderson et al. 1997; Cheriaa and Bakhrouf 2009; Jang et al. 2004; Jones and Falkinham III 2003; Li et al. 2009; Ren et al. 2006). Analysis of *Clostridium perfringens*, *Aeromonas hydrophila* DN322, *Citrobacter* sp. KCTC 18061P, *Pseudomonas* sp. MDB-1, and *Lactobacillus acidophilus* cultures incubated with triphenylmethane dyes showed that these dyes were converted to leuco derivatives (Henderson et al. 1997; Ren et al. 2006; Jang et al. 2004; Li et al. 2009). These leuco derivatives, also as contaminants, were equally toxic to triphenylmethane dyes and were eliminated much slower than dyes from environment (Burchmore and Wilkinson 1993). However, biodegradation of these leuco derivatives was hardly reported previously, even in triphenylmethane dyes decolorizing bacteria except *Pseudomonas* sp. MDB-1. Therefore, much work is still required to isolate microorganisms applicable to biodegradation of leuco derivatives of triphenylmethane dyes and to study their physiology, in order not only to understand the mechanisms underlying leuco derivatives biodegradation, but also for biotechnological purposes.

In this present study, we reported the isolation and characterization of a bacterium, *Sphingomonas* sp. CM9, capable of efficiently degrading leuco derivatives of triphenylmethane dyes.

Materials and methods

Dyes and chemicals

Triphenylmethane dyes malachite green (MG), crystal violet (CV) and basic fuchsin (BF) were purchased from Shanghai Sangon Co, Ltd, China. Leucomalachite green (LMG) and leucocrystal violet (LCV) were purchased from Sigma-Aldrich. All other chemicals used were of analytical grade.

Isolation and identification of LMG degrading bacterium

A LMG-degrading bacterium designated CM9 was isolated from an aquafarm field where MG had been repeated applied for more than 8 years. The identification of CM9 was performed according to Bergey's manual of determinative bacteriology and its 16S rRNA gene sequence. Genomic DNA from CM9 strain was extracted by the method of high salt concentration precipitation (Miller et al. 1988). The 16S rRNA gene was amplified by using two universal primers, 27F (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and 1495R (5′-CTA CGG CTA CCT TGT TAC GA-3′) (Weisburg et al. 1991) and ligated with pMD19-T (TaKaRa Biotechnology, Dalian, China), then transformed into *Escherichia coli* DH5α. The sequence of the amplified 16S rRNA gene was determined by Shanghai Sangon Biotechnology. 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).

Biochemical synthesis of leucobasic fuchsin (LBF)

The specific triphenylmethane reductase (TMR) gene from *Listeria monocytogenes* str. 4b H7858, sharing 100% nucleotide identity with the *tmr* gene from *Citrobacter* sp. KCTC 18061P, was cloned into plasmid pUC119 and expressed in *E. coli* BL21 (DE3) as described previously by Jang et al. (2005). The His-tagged TMR was purified by a 2-ml volume of NTA-Ni^{2+} agarose (QIAGEN) at 4°C and stored in the 50 mM sodium phosphate buffer (pH 7.5) (Jang et al. 2005). Five milligrams of BF and 50 ml of 50 mM sodium phosphate buffer (pH 7.5) were equilibrated at 30°C in a water-bath shaker, respectively. The enzymatic reaction was performed by adding 1 ml of the purified His-tagged TMR (final

concentration, 2 mg l^{-1}) with 100 μM NADH at 40°C for 20 min and stopped by addition of twice volume of acetonitrile. LBF was purified by preparative liquid chromatography with a C_{18} preparative column (10 mm × 250 mm, BonChrom) respectively. Water (20%) in acetonitrile was used as the mobile phase.

Degradation of LMG, LCV and LBF by growing cells

The degradation of LMG, LCV and LBF by strain CM9 was carried out in mineral salt medium (SM) containing (per liter) 1.0 g of NH_4NO_3, 1.0 g of NaCl, 1.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, and 0.1 g of $MgSO_4$. Cells of CM9 grown overnight in LB medium (containing (per liter) 10.0 g of NaCl, 10.0 g of Tryptone and 5.0 g of yeast extract) were collected by centrifugation at 12,000×*g* in microcentrifuge tubes at room temperature for 2 min, washed 3 times with sterile water and resuspended in equal volume of SM medium. These precultures were then transferred at 2% (vol/vol) to fresh SM medium containing 100 mg l^{-1} of each leuco derivative. Each flask containing derivative was incubated at 30°C on a rotary shaker at 200 rpm. Appropriate controls containing medium plus the 100 mg l^{-1} of each leuco derivative were prepared simultaneously. Aliquots (500 μl) were taken out periodically and added twice volume (1000 μl) of acetonitrile. The mixture was vortexed for 1.0 min. After centrifugation at 12,000×*g* for 5.0 min, the supernatant was collected and analyzed by high performance liquid chromatography/mass spectrometry (HPLC/MS) or by gas chromatography/mass spectrometry (GC/MS). Simultaneously, equivalent aliquots (500 μl) were also taken out periodically and the cell density at OD_{600nm} was measured by UV–Vis spectrophotometer UV-2000.

Decolorization of MG, CV and BF by growing cells

The decolorization of triphenylmethane dyes by strain CM9 was also carried out. The procedure was as described above. The only difference was addition of 100 mg l^{-1} of each triphenylmethane dye in medium instead of leuco derivative.

Crude extract preparation

One hundred milliliter precultured CM9 cells were centrifuged at 12,000×*g* for 5 min. The precipitated cells were washed twice with sterile water and then suspended in 30 ml of 50 mM sodium phosphate buffer (pH 7.5). Cells were disrupted by sonication (VCX600; Sonics, Newtown, CT, USA) on ice and centrifuged at 20,000×*g* for 20 min. The supernatant was stored at 4°C.

Effects of pH, temperature and metal ions on degradation of LMG by CM9 crude extract

The effect of pH on degradation activity was determined by incubating the crude extract with 3 mg l^{-1} LMG for 5 min at pH values in the range from pH 4 to 10 at 30°C. The effect of temperature was determined in pH 7.0 at the range from 10 to 80°C for 5 min. The effect of metal ions was determined by addition of various metal salts to the reaction mixture and incubation at 30°C for 5 min. The residual LMG was detected by HPLC/MS.

Degradation assay

Reversed-phase HPLC/MS with electrospray ionization (ESI) was performed on a Finnigan LCQ Advantage Max instrument under standard positive-ion electrospray conditions. HPLC separation was performed on a C_{18} 5 μm column, 150 mm × 4.6 mm ID (Shimadzu Technologies, Tokyo, Japan). The mobile phase was 50% acetonitrile in H_2O (0.1% formic acid) for 2 min followed by a 10 min linear gradient to 95% acetonitrile (0.1% formic acid), where it was held for an additional 10 min with a flow rate of 0.35 ml min^{-1}. Positive ions were acquired in full scan m/z 50-600 for identification in 1 s scan time.

Identification of degradation products of LMG by CM9

GC/MS analysis was performed with a Varian-Saturn GC/MS (Varian-Saturn 2200), equipped with the CP-3800 GC, injection port split-splitless, and the 2000 Series Ion Trap MS. The column used was a DB-1701 Low Bleed/MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent). The oven temperature was programmed as follows: hold time at 100°C,

1 min; ramp rate at 10°C min^{-1} to 310°C; hold time at 310°C, 10 min. The temperatures corresponding to the transfer line and the ion trap were 290 and 250°C, respectively, and the ionization energy was 70 eV. The injection volume was 1 µl via a splitless injection at 290°C. Helium was used as a carrier at a flow rate of 1.0 ml/min.

Results

Isolation and identification of a LMG-degrading bacterium CM9

Bacterium CM9 was isolated for its relatively high LMG degrading efficiency. The colony morphology of strain CM9 on the LB plate was yellowish, smooth, and wet. This strain was Gram-negative, rod-shaped, strictly aerobic and motile with a single polar flagellum. CM9 was naturally resistant to 50 mg l^{-1} streptomycin and sensitive to ampicillin (10 mg l^{-1}), gentamycin (10 mg l^{-1}), and kanamycin (10 mg l^{-1}). The physiologic characteristics of CM9 were determined by conventional biochemical tests. The bacteria tested positive for catalase, oxidase, nitrate reduction and poly-hydroxybutyrate but tested negative for starch hydrolysis, fructose fermentation, Voges-Proskauer test. The 16S rRNA gene sequence of CM9 was deposited at GenBank under Accession No. HQ116524. Comparison with available sequences in GenBank showed a high similarity of CM9 with species in the genus *Sphingomonas*. A phylogenetic tree based on known representatives of *Sphingomonas* species is presented in Fig. 1. On the basis of the above characters, strain CM9 was given a preliminary identification as a *Sphingomonas* sp.

Degradation of LMG, LCV and LBF by CM9

Strain CM9 completely degraded 100 mg l^{-1} of each leuco derivative in 24 h. The concentration of residual leuco derivatives versus time was shown in Fig. 2. The complete degradation of LMG was observed in 10 h, followed by LBF in 12 h. The degradation of LCV was the slowest. After 18 h, approximately 5 mg l^{-1} of LCV was still detected, but 24 h the residual LCV was degraded completely by CM9. No disappearance of all leuco derivatives

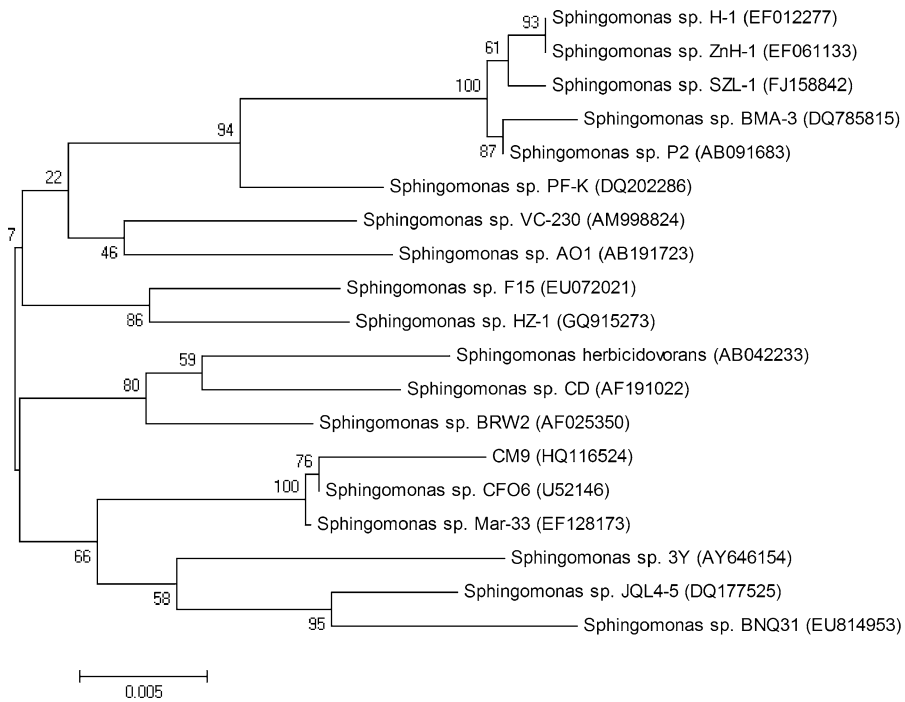

Fig. 1 Phylogenetic tree illustrating the 16S rDNA gene similarity of CM9 to strains exhibiting highest sequence similarity (RDP analysis and FASTA)

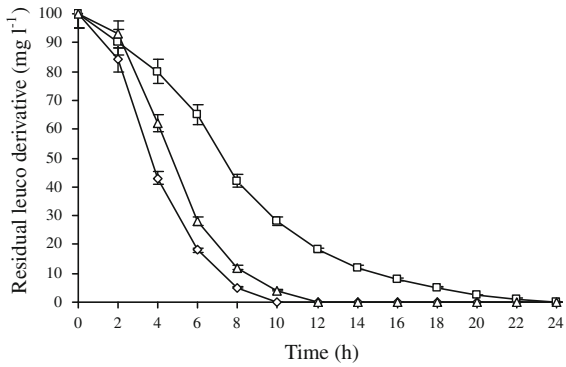

Fig. 2 Degradation of leuco derivatives of triphenylmethane dyes by *Sphingomonas* sp. CM9 in SM. (*open diamond*) LMG; (*open triangle*) LBF; (*open square*) LCV; the *error bars* indicate standard deviations

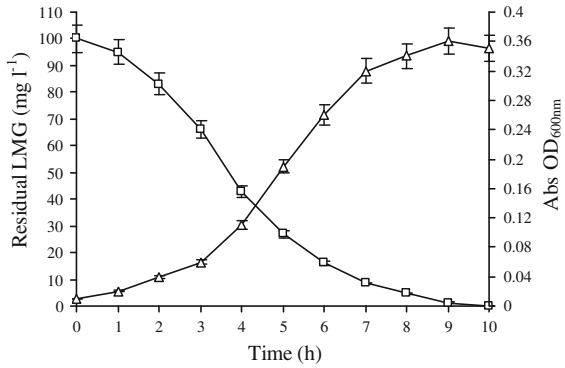

Fig. 3 Degradation of LMG by strain CM9 with companying data for cell growth in SM medium; the *error bars* indicate standard deviations

was detected in control cultures. The cell mass of CM9 was measured after addition of 100 mg l^{-1} LMG. CM9 cells continued to proliferate in the presence of LMG, with an exponential phase of growth occurring between 3 and 7 h (Fig. 3). No growth occurred in cultures lacking LMG. The growth of strain CM9 and the complete disappearance of LMG demonstrated that the bacteria could effectively use LMG as sole source of carbon for growth.

Decolorization of MG, CV and BF by CM9

Strain CM9 could also decolorized 100 mg l^{-1} of MG, CV and BF in 14 h, respectively. The concentration of residual triphenylmethane dyes versus time was shown in Fig. 4. CM9 showed very strong decolorization activity against MG. After 8 h, MG was decolorized completely. A small amount of LMG was cumulated during decolorization, but it was rapidly degraded later. The decolorization of BF was slower than that of MG, but faster than that of CV. Nearly 20 mg l^{-1} LBF was detected from medium after 8 h and it disappeared completely after 14 h. The decolorized CV was the slowest. After 14 h, CM9 decolorized 100 mg l^{-1} of CV completely. However, there was 43 mg l^{-1} LCV in medium in that time, which eventually disappeared after 26 h.

Effects of pH, temperature and metal ions on degradation of LMG by CM9 crude extract

The LMG degradation activity of CM9 crude extract was detected in a wide pH range (5.0–9.0), with an optimum at pH 7.0, but it sharply reduced at pH values beyond 6.0 and 9.0. The optimal reaction temperature was observed to be 30°C. The crude extract completely lost 80% activity when temperature was higher than 50°C and was completely inactivated at 70°C. CM9 crude extract was strongly inhibited with many metal ions (Ag^{+}, Ca^{2+}, Ba^{2+}, Mg^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, and Zn^{2+}) (0.2 mM) and little inhibition effect on the degradation activity was observed (less than 5%).

Identification of degradation products of LMG by CM9

GC/MS was employed to detect the degradation products of LMG by growing CM9 cells. Two degradation products, designated M1 and M2, were detected at 3.215 and 5.858 min, respectively, as shown in the total-ion chromatogram (Fig. 5a). The mass spectrum of M1 showed a molecular ion peak *m/z* 92 ($M1^{+}$) and major fragment peaks such as *m/z* 91 ($M2^{+}$–H) and *m/z* 65 ($M2^{+}$–H–C_2H_2) (Fig. 5b), which were identical to those observed in the spectrum of authentic methylbenzene. So M1 was identified as methylbenzene. The mass spectrum of M2 showed a molecular ion peak *m/z* 109 ($M2^{+}$) and major fragment peaks such as *m/z* 80 ($M2^{+}$-H–CO) (Fig. 5c), which were identical to those observed in the spectrum of authentic 4-aminophenol. From these results, M2 was identified as 4-aminophenol.

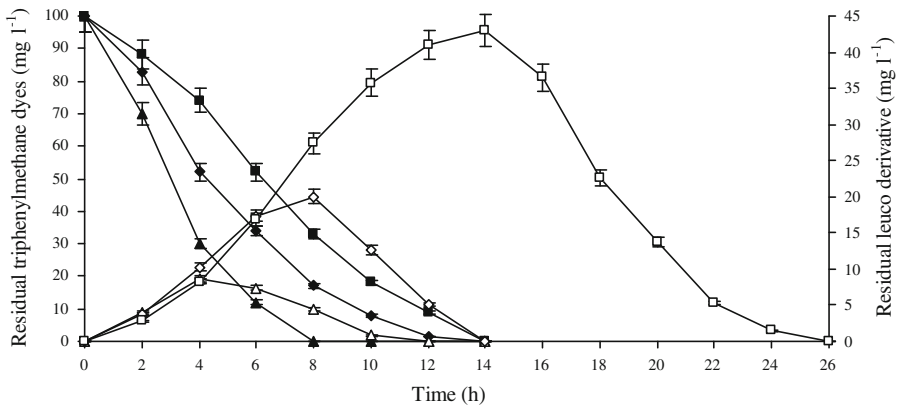

Fig. 4 Degradation of 100 mg l^{-1} triphenylmethane dyes by CM9. (*filled triangle*) MG; (*filled diamond*) BF; (*filled square*) CV; (*open triangle*) cumulated LMG in medium; (*open diamond*) cumulated LBF in medium; (*open square*) cumulated LCV in medium; the *error bars* indicate standard deviations

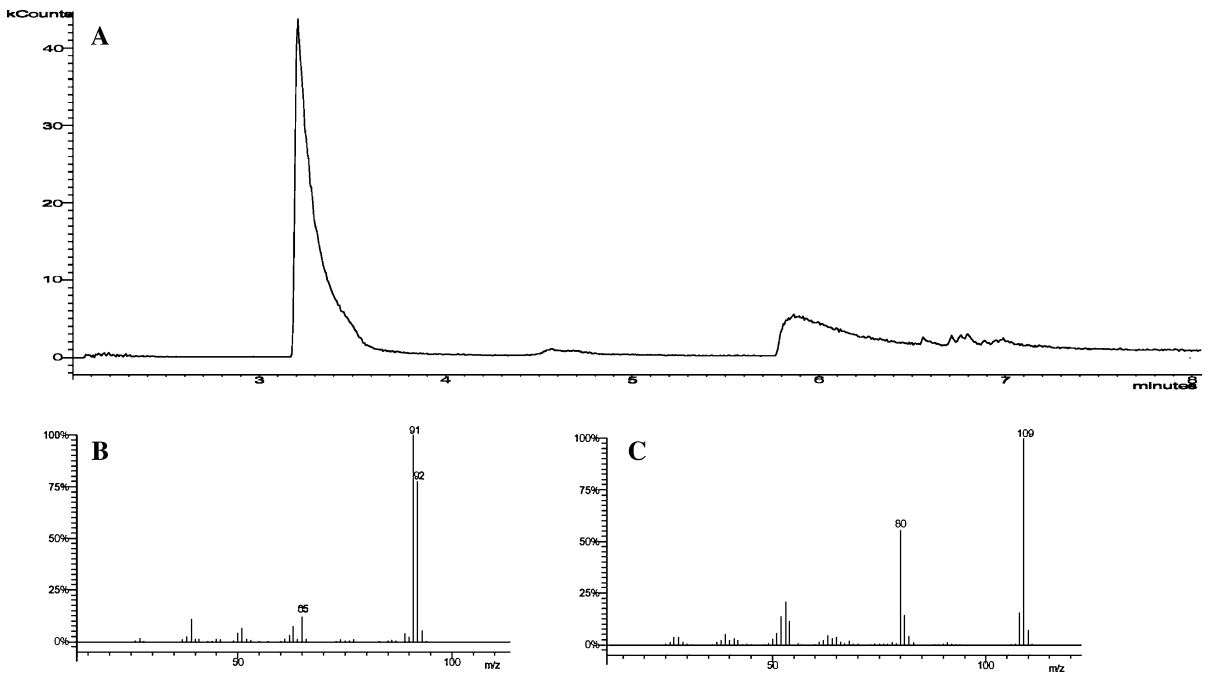

Fig. 5 GC/MS spectral analysis of an extract from a culture of CM9 dosed with LMG. **a** Total-ion chromatogram; **b** Mass spectra of metabolite M1; **c** Mass spectra of metabolite M2

Discussion

In the present study, a leuco derivatives of triphenylmethane dyes degrading bacterial strain CM9 was isolated and identified as a member of the *Sphingomonas* genus by the analysis of physiological and biochemical characteristics and its 16S rRNA gene sequence. CM9 could completely degrade LMG, LCV and LBF rapidly and also had strong decolorization activity of MG, CV and BF. Most of the previously reported triphenylmethane dyes degrading strains have strong decolorization activity (Henderson et al. 1997; Jang et al. 2004; Jones and Falkinham III 2003; Li et al. 2009; Ren et al. 2006). However, strong degradation activity of leuco derivatives was hardly reported in these strains except *Pseudomonas* sp. MDB-1. These leuco derivatives as contaminants were persistent in environment for a longer time. The excellent degradation ability of CM9 suggested that this strain had potential applications in the treatment of wastewater from the dye and aquaculture industries.

In recent years, the biochemical mechanism underlying the decolorization has been elucidated in some bacteria. The specific decolorization enzyme TMR was purified and characterized from *Citrobacter* sp. KCTC 18061P (Jang et al. 2005). TMR catalyzed the NADH 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 (Jang et al. 2005). The *tmr* gene has also been cloned from many strains as *Aeromonas hydrophila* DN322, *Pseudomonas* sp. MDB-1 and an IncP-1β plasmid pGNB1 from bacteria community from the activated sludge compartment (Li et al. 2009; Ren et al. 2006; Schlüter et al. 2007). However, in our study, no *tmr* gene or *tmr*-like gene was found in CM9 (data not shown). HPLC/MS analysis showed CM9 decolorized MG, CV and BF to form LMG, LCV and LBF, respectively. These results indicated that maybe some novel protein instead of TMR was responsible for the decolorization of dyes in CM9 strain.

Although we have known triphenylmethane dyes could be converted to leuco derivatives via reduction reactions, degradation process of these leuco derivatives in environment was poorly understood. In 2001, proposed mechanism for the metabolism of MG and LMG in filamentous fungus *Cunninghamella elegans* ATCC 36112 was reported (Cha et al. 2001). LMG was converted to *N*-demethylated and *N*-oxidized metabolites, including primary and secondary arylamines in ATCC 36112. However, the metabolism of LMG in bacteria was still unknown. In this study, two degradation products of LMG, methylbenzene and 4-aminophenol, were identified by GC/MS during incubation with CM9. Although we did not know the detailed degradation pathway from LMG to 4-aminophenol and methylbenzene, the main structure of triphenylmethane was doubtless broken by CM9. Obviously, *N*-demethylation reaction also occured in this pathway, but no *N*-demethylated-LMG was detected by HPLC/MS and GC/MS (data not shown). So we could not confirm *N*-demethylation reaction took place before triphenylmethane broken reaction. Much work is required to elucidate the degradation mechanism of LMG and identify the metabolic derivatives.

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