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

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**Schedule**

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**Keywords (separated by ' - '):** Leuco derivatives of triphenylmethane dyes - Degradation - Decolorization - Metabolic products
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Biodegradation of leuco derivatives of triphenylmethane dyes by *Sphingomonas* sp. CM9

Jun Wu · Liguan Li · Hongwei Du · Lijuan Jiang · Qiong Zhang · Zhongbo Wei · Xiaolin Wang · Lin Xiao · Liuyan Yang

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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

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 1996, 1999).
Materials and methods

Dyes and chemicals

Triphenylmethane dyes malachite green (MG), crystal violet (CV) and basic fuchsin (BF) 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 aqua farm field where MG had been repeatedly 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-3’) and 1495R (5’-CTA CGG CCT 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-Ni2+ 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
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 NH₄NO₃, 1.0 g of NaCl, 1.5 g of K₂HPO₄, 0.5 g of KH₂PO₄, and 0.1 g of MgSO₄. 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 for 5 min. After centrifugation at 20,000×g on ice and centrifuged at 20,000×g for 5 min, the supernatant was stored at 4°C.

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 C₁₈ 5 μm column, 150 mm × 4.6 mm ID (Shimadzu Technologies, Tokyo, Japan). The mobile phase was 50% acetonitrile in H₂O (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)](image-url)
280 was detected in control cultures. The cell mass of 281 CM9 was measured after addition of 100 mg l\(^{-1}\) 282 LMG. CM9 cells continued to proliferate in the 283 presence of LMG, with an exponential phase of 284 growth occurring between 3 and 7 h (Fig. 3). No 285 growth occurred in cultures lacking LMG. The 286 growth of strain CM9 and the complete disappear- 287 ance of LMG demonstrated that the bacteria could 288 effectively use LMG as sole source of carbon for 289 growth.

290 Decolorization of MG, CV and BF by CM9

291 Strain CM9 could also decolorized 100 mg l\(^{-1}\) of 292 MG, CV and BF in 14 h, respectively. The concentra- 293 tion of residual triphenylmethane dyes versus time 294 was shown in Fig. 4. CM9 showed very strong 295 decolorization activity against MG. After 8 h, MG 296 was decolorized completely. A small amount of LMG 297 was cumulated during decolorization, but it was 298 rapidly degraded later. The decolorization of BF was 299 slower than that of MG, but faster than that of CV. 300 Nearly 20 mg l\(^{-1}\) LBF was detected from medium 301 after 8 h and it disappeared completely after 14 h. The 302 decolorized CV was the slowest. After 14 h, CM9 303 decolorized 100 mg l\(^{-1}\) of CV completely. However, 304 there was 43 mg l\(^{-1}\) LCV in medium in that time, 305 which eventually disappeared after 26 h.

306 Effects of pH, temperature and metal ions 307 on degradation of LMG by CM9 crude extract

The LMG degradation activity of CM9 crude extract 308 was detected in a wide pH range (5.0–9.0), with an 309 optimum at pH 7.0, but it sharply reduced at pH 310 values beyond 6.0 and 9.0. The optimal reaction 311 temperature was observed to be 30°C. The crude 312 extract completely lost 80% activity when tempera- 313 ture was higher than 50°C and was completely 314 inactivated at 70°C. CM9 crude extract was strongly 315 inhibited with many metal ions (Ag\(^{+\},\) Ca\(^{2+\},\) Ba\(^{2+\},\) 316 Mg\(^{2+\},\) Ni\(^{2+\},\) Cu\(^{2+\},\) Hg\(^{2+\},\) and Zn\(^{2+\})\) (0.2 mM) and 317 little inhibition effect on the degradation activity was 318 observed (less than 5%).

319 Identification of degradation products of LMG 320 by CM9

GC/MS was employed to detect the degradation 321 products of LMG by growing CM9 cells. Two 322 degradation products, designated M1 and M2, were 323 detected at 3.215 and 5.858 min, respectively, as 324 shown in the total-ion chromatogram (Fig. 5a). The 325 mass spectrum of M1 showed a molecular ion peak 326 \(m/z\) 92 (M1\(^{+\})\) and major fragment peaks such as 327 \(m/z\) 91 (M2\(^{+}\)–H) and \(m/z\) 65 (M2\(^{+}\)–H–C\(_2\)H\(_2\)) 328 (Fig. 5b), which were identical to those observed in 329 the spectrum of authentic methylbenzene. So M1 was 330 identified as methylbenzene. The mass spectrum of 331 M2 showed a molecular ion peak \(m/z\) 109 (M2\(^{+\})\) and 332 major fragment peaks such as \(m/z\) 80 (M2\(^{+}\)–H–CO) 333 (Fig. 5c), which were identical to those observed in 334 the spectrum of authentic 4-aminophenol. From these 335 results, M2 was identified as 4-aminophenol.
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 Falkingham 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 aryamines 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 occurred 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.

**Acknowledgments** This work was granted by the National Natural Science Foundation (Projects no. 20637030), the National Science and Technology Supporting Item (Projects no. 2006BA08B01-02) and the Science and Technology Department of Jiangsu Province of The People’s Republic of China (Project no. BS2007160). We thank Jianfang Feng and Lianhong Wang for technical assistance.

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