Journal of Life Science **2004**, Vol. 14. No. 1. 21~25 ©JLS

Identification of Genes Involved in Decolorization of Crystal Violet and Malachite Green in *Citrobacter* sp.

Young-Mi Lee, Moon-Sun Jang, Seok-Jo Kim, Yong-Lark Choi, Young-Su Cho and Young-Choon Lee*

Faculty of Biotechnology, College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Korea

Received August 30, 2003 / Accepted November 5, 2003

To identify genes involved in the decolorization of both crystal violet and malachite green, we isolated random mutants generated by transposon insertion in triphenylmethane-decolorizing bacterium, *Citrobacter* sp. The resulting mutant bank yielded 14 mutants with complete defect in color removal capability of both crystal violet and malachite green. Southern hybridization with a Tn5 fragment as a probe showed a single hybridized band in 5 mutants and these mutants appeared to have insertions at different sites of the chromosome. Tn5-inserted genes were isolated and the DNA sequence flanking Tn5 was determined. From comparison with a sequence database, putative protein products encoded by *cmg* genes were identified as follows. *cmg* 2 is MalG protein in maltose transport system; *cmg* 6 is transcriptional regulator (LysR-type); *cmg* 12 is a putative oxidoreductase. The sequences deduced from two *cmg* genes, *cmg* 8 and *cmg* 11, showed no significant similarity to any protein with a known function. Therefore, these results indicate that these two *cmg* genes encode unidentified proteins responsible for decolorization of both crystal violet and malachite green.

Key words – Transposon mutagenesis; Decolorization; Triphenylmethane dye; *Citrobacter* sp.

Triphenylmethane dyes are used extensively in textile industries for dying nylon, wool, silk, cotton [19], and significantly some dyes have been shown to be a mutagen, a mitotic poison and clastogen [3,4]. The degradation of dyes has received consideable attention from the viewpoint of treating industrial wastewater containing dyes. Studies on the biodegradation of triphenylmethane dyes have focused primarily on the decolorization of dyes via reduction reactions [5].

Crystal violet (CV) is a triphenylmethane dye that has been used extensively in human and veterinary medicine as biological stain [24,25], and shown to inhibits glutathione S-transferases from both insect sources [6] and from rat liver [15]. Also, CV has been suggested to be responsible for promotion of tumor growth in some species of fish [27]. Recently, CV exhibited pronounced phototoxicity toward L1210 leukemia cells but comparatively small toxic effects toward normal hematopoietic cells [22]. Malachite green (MG) is also a triphenylmethane dye that has been widely used to prevent fungal infections in the fish farming industry [1,13]. MG is highly toxic to mammalian cells; it promotes hepatic tumor formation in rodents and also causes reproductive abnormalities in rabbits and fish [17,28]. MG is reductively decolorized by intestinal microflora from a variety of animals [21], and mycobacterial strains [23].

Several triphenylmethane dye-degrading microorganisms have been reported and their characteristics have been reviewed recently [5,7]. Most of these microorganisms decolorized CV and MG. The mechanism for biodecolorization of CV and MG has been elucidated by fungi [5, 10,32,33], but not by bacteria. CV and MG were degraded by ligninolytic culture of *Phanerochaete chrysosporium*, and its initial oxidation proceeds via N-demethylation catalyzed by lignin peroxidase [10]. Also, Decolorization of CV was found to be carried out by laccase in extracellular fluid from *Cyathus bulleri* [33], and by peroxidase from *Pleurotus ostreatus* [32].

Very recently, we isolated a new potent bacterium, *Citrobacter* sp. having a higher decolorization capability, even at a high concentration of triphenylmethane and azo dyes than any microorganisms reported so far [2]. Decolorization by this strain was shown to be performed by the extracellular enzymatic system clearly different from that observed in ligninolytic cultures of fungi.

To understand the molecular mechanism of biodecolorization by bacteria, we used transposon mutagenesis with Tn5 in investigating genes responsible for decolorization of both CV and MG in *Citrobacter* sp. In this study, we isolated six decolorizing-defective mutants and characterized their genes.

*Corresponding author
Tel : +82-51-200-7591, Fax : +82-51-200-6993
E-mail : yclee@daunet.donga.ac.kr

Materials and Methods

Bacteria and growth condition

Escherichia coli JM109 was used as a host for cloning and sequencing. *E. coli* MC1061[pR388(ts)::Tn5] was kindly provided by Dr. C. Sasakawa (Institute of Medical Science, The University of Tokyo, Japan) and used as a Tn5 donor in transposon mutagenesis [30]. *E. coli* MC1061[pR388(ts):: Tn5] was grown at 30℃ in LB medium containing trimethoprim (Tp, 12.5 μg/ml) and kanamycin (50 μg/ml). *Citrobacter* sp. was grown at 37℃ in LB medium containing streptomycin (50 μg/ml).

Transposon mutagenesis

E. coli MC1061[pR388(ts)::Tn5] and *Citrobacter* sp. were grown to an OD_{660nm} of 0.6, 500 ml of each strain were taken out, mixed and 30 ml of mixture was spotted onto LB agar plates. After incubation at 30℃ for 12 h, cells were collected by scraping and transconjugants were selected after incubation at 30℃ for 24 h on M9 agar plates containing Tp (12.5 μg/ml) and streptomycin (50 μg/ml). From transconjugants strains lacking pR388(ts)::Tn5 were isolated by incubation at 42℃ for 15 h in LB agar plate containing kanamycin (50 μg/ml). Colonies were picked and transferred onto LB agar plate containing kanamycin (50 μg/ml) and CV (245 μM). After incubation at 37℃ for 3 days, colonies lacking decolorization activity were selected and confirmed by shaking incubation in LB medium containing kanamycin (50 μg/ml) and CV (245 μM). After strains defective in decolorization of CV were ransferred onto LB agar plate containing kanamycin (50 μg/ml) and MG (200 μM). After incubation at 37℃ for 3 days, colonies lacking decolorization of MG were selected and confirmed by shaking incubation in LB medium containing kanamycin (50 μg/ml) and MG (200 μM). Also, it was confirmed, by Southern hybridization [29] with Tn5 DNA fragment as a probe, that these dye-nondecolorizing cells were derived from *Citrobacter* sp. by Tn5 insertion.

Recombinant DNA techniques and Southern blot analysis

Standard recombinant DNA procedures were used [29]. Chromosomal DNAs of *Citrobacter* sp. and mutant strains were prepared by the method of Marmur [26]. Chromosamal DNAs were digested with *Eco*RI for detection of single insertion of Tn5, and with various enzymes for subcloning. Digests were run on 0.5 % SeaKem GTG agarose gel (FMC, USA) at 24 V for 17 h. Southern analysis was carried out as detailed in Sambrook *et al.* [29]. Blots on Hybond-N^+ membrane (Amersham Pharmacia Biotech., Sweden) were probed and developed with ^{32}P-labelled Tn5 probes which were prepared using the random primer labelling kit (Amersham Pharmacia Biotech., Sweden). The nylon membrane was autoraidographed with X-ray film (Kodak Co., USA).

Identification of *cmg* genes

DNA fragments containing Tn5 DNA fragment confirmed by Southern blot analysis were subcloned into the corresponding sites of pBluescript SK (+) or pUC 118 and 119. The DNA sequence flanking Tn5 was determined by using a synthetic primer (5′-CTGTCTCTTGATCAGATCT-3′) complementary to the distal end of Tn5. The obtained sequence was translated; amino acid sequences inferred from each open reading frame which had been interrupted by Tn5 were compared with protein sequences in the database by using BLAST, and the function of each *cmg* gene was deduced from the similarity of its product to known proteins.

Results and Discussion

Isolation of mutants defective in decolorization of both CV and MG

To investigate the molecular mechanism of dye decolorization, we tried to isolate mutants defective in decolorization of both CV and MG by transposon mutagenesis. Transposon mutagenesis was carried out by conjugative transfer of Tn5 from *E. coli* MC1061[pR388(ts)::Tn5] to *Citrobacter* sp., and mutants defective in decolorization of both CV and MG were screened by replica plating on the plates containing CV followed by MG. From a screening of about 25,000 colonies, we isolated 14 mutants that did not decolorize both CV and MG. These mutants should be affected in dye-decolorizing genes, and they were called *cmg* mutants. Southern hybridization [29] of *Eco*RI-digested chromosomal DNA fragments using Tn5 DNA fragment as a probe showed a single hybridized band in 8 mutants, revealing that they were single transposon insertion. Other mutants had two or three transposon insertions in the genome. Restriction patterns and sequence comparisons showed that 3 of 8 mutants had transposon insertions in

Table 1. Characterization of the decolorization-defective mutants for both CV and MG with single transposon insertion

Mutants	Homologous protein[a] (% identity)	Organism	Putative function or location
cmg 2	MalG (50)	*Escherichia coli* O157:H7	Transport system
cmg 6	NP_418042 (93)	*Escherichia coli* K-12	Transcriptional regulator (LysR-type)
cmg 8	NP_415105 (89)	*Escherichia coli* K-12	Unknown (hypothetical)
cmg 11	NP_415882 (90)	*Escherichia coli* K-12	Unknown (hypothetical)
cmg 12	P76440 (83)	*Escherichia coli* K-12	Oxidoreductase

[a]Homology based on BLAST program of the National Center for Biotechnology Information (NCBI). The homologue with the highest sequence homology is shown. In the absence of a clear protein designation, homologues are listed by accession numbers.

identical positions. Therefore, as shown in Fig. 1, 5 mutants harboring single transposon insertion at different sites of the chromosome were characterized further.

Identification of *cmg* genes

To elucidate possible functions of *cmg* genes, we cloned the Tn5-inserted genes and the DNA sequence flanking the transposon was determined. The possible function of each *cmg* gene was inferred from a comparison of the translated amino acid sequences in a database.

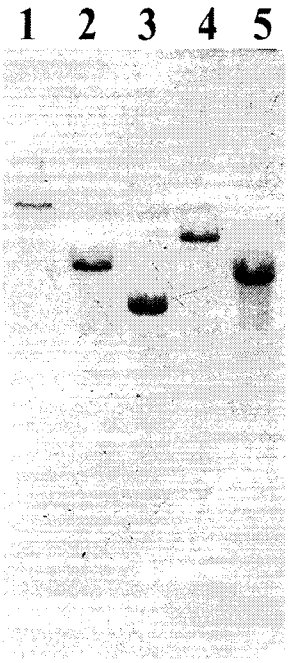

Fig. 1. Southern blot analysis with Tn5 DNA as a probe. Chromosomal DNA was isolated from each strain, digested with *Eco*RI, and electrophoresed on a 0.5 % (w/v) agarose gel. DNA was blotted on a nylon membrane and hybridized with ^{32}P-labeled Tn5 DNA as a probe. Lanes 1 to 5, chromosomal DNAs isolated from the mutant strains: Cmg 2 (lane 1), Cmg 6 (lane 2), Cmg 8 (lane 3), Cmg 11 (lane 4) and Cmg 12 (lane 5).

The amino acid sequence deduced from the *cmg* 2 showed 50% identity with MalG protein in maltose transport system of *E. coli* [14]. In *E. coli*, MalG and MalF are hydrophobic inner membrane components mediating the energy-dependent translocation of substrate into the cytoplasm [14]. The binding protein-dependent maltose transport system of enterobacteria (MalFGK2), a member of the ABC transport superfamily, is composed of two intergal membrane proteins, MalF and MalG, and of two copies of an ATPase subunit, MalK, which hydrolyze ATP, thus energizing the translocation process [9]. From these observations, it is highly probable that *cmg* 2 is the gene encoding protein acting as transporter associated with the decolorization of CV and MG.

Sequence analyses of flanking regions of the transposons in *cmg* 6 showed 93% identity to a putative LysR-type transcriptional regulators, respectively, of *E. coli* K-12 [8]. The success of aromatic catabolic pathway depends on two major elements: the catabolic enzymes leading to mineralization of the compound; and the regulatory elements [16]. Regulatory proteins and regulated promoters are the key elements that control the transcription of catabolic operons. LysR-type regulatory protein is transcriptional regulator that also control the expression of aromatic catabolic pathways [18,31]. Thus, we predict that *cmg* 6 may the gene encoding regulatory protein that control the transcription of genes associated with catabolism of CV and MG in *Citrobacter* sp.. We isolated about a 6 kb fragment from *Citrobacter* sp. by Southern hybridization using *cmg* 6 as a probe and found a gene cluster comprised four open reading frames (ORFs) by restriction mapping and partial sequencing (unpublished data).

The amino acid sequences deduced from the DNA sequences of *cmg* 12 had significant similarity (83% identity) to the sequence of a putative oxidoreductase of *E. coli* K-12 [39]. CV and MG can be reductively decolorized [12,20].

The enzymatic reduction of MG to leuco MG by intestinal microflora results in dye decolorization [21]. Decolorization and biodegradation of CV and MG by a fungus *Phanerochaete chrysosporium* occur by oxidative reaction via *N*-demethylation catalyzed by lignin peroxidase [10]. *P. chrysosporium* could also degrade these dyes under nonligninolytic conditions, suggesting that another mechanism for degrading these dyes existed in this fungus [10]. These reports strongly suggest that *cmg* 12 may encode protein responsible for degradation of CV and MG in *Citrobacter* sp..

Sequence analyses of *cmg*s 8 and 11 showed no significant similarity to any protein with a known function, but did show more than 89% homology with hypothetical proteins of *E. coli*.

On the basis of these results, the cloning, sequencing and expression of Tn5-inserted genes should help to clarify the exact functions of their genes responsible for the decolorization of CV and MG. In our laboratory, cloning of open reading frame (ORF) of these genes using fragments of *cmg* genes obtained in this study are in progress.

Acknowledgements

This work was supported by the 21C Frontier Microbial Genomics and Applications Center Program (Grant MG02-0301-005-2-2-0) from Ministry of Science & Technology, Republic of Korea.

References

1. Alderman, D. J. 1985. Malachite green: a review. *J. Fish. Dis.* **8**, 289-298.
2. An, S.-Y., K.-W. Kim, Y.-L. Choi, Y.-S. Cho, S.-K. Min, I.-H. Cha, C.-H. Kim and Y.-C. Lee. 2000. Microbial decolorization of triphenylmethane and azo dyes by *Citrobacter* sp. *Enzyme Microb. Technol.* submitted and in review.
3. Au, W., S. Pathak, C. J. Collie and T. C. Hsu. 1978. Cytogenetic toxicity of gentian violet and crystal violet on mammalian cells *in vitro*. *Mutat. Res.* **58**, 269-276.
4. Au, W., M. A. Butler, S. F. Bloom and T. S. Matmey. 1979. Further study of the genetic toxicology of gentian violet. *Mutat. Res.* **66**, 103-112.
5. Azmi, W., R. K. Sani and U. C. Banerjee. 1998. Biodegradation of triphenylmethane dyes. *Enzyme Microb. Technol.* **22**, 185-191.
6. Balabaskaran, S. and J. N. Smith. 1970. The inhibition of 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane (DDT) dehydrochlorinase and glutathione S-aryltransferase in grass-grub and housefly preparations. *Biochem. J.* **17**, 989-996.
7. Banat, I. M., P. Nigam, D. Singh and R. Marchant. 1996. Microbial decolorization of textile-dye-containing effluents: a review. *Biores. Technol.* **58**, 217-227.
8. Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453-1474.
9. Boos, W. and H. Shuman. 1998. Maltose/maltodextrin system of *Escherichia coli*: transport, metabolism, and regulation. *Microbiol. Mol. Biol. Rev.* **62**, 204-229.
10. Bumpus, J. A. and B. J. Brock. 1988. Biodegradation of crystal violet by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **54**, 1143-1150.
12. Cornish, H. H., E. Zamora and R. E. Bahor. 1964. Metabolism of triphenylmethane and triphenylcarbinol. *Arch. Biochem. Biophys.* **107**, 319-324.
13. Culp, S. J. and F. A. Beland. 1996. Malachite green: a toxicological review. *J. Am. Coll. Toxicol.* **15**, 219-238.
14. Dassa, E. and S. Muir. 1993. Membrane topology of MalG, an inner membrane protein from the maltose transport system of *Escherichia coli*. *Mol. Microbiol.* **7**, 29-38.
15. Debnam, P., S. Glanville and A. Clark. 1993. Inhibition of glutathione S-transferases from rat liver by basic triphenylmethane dyes. *Biochem. Pharmacol.* **45**, 1227-1233.
16. de Lorenzo, V. and J. Pérez-Martín. 1996. Regulatory noise in prokaryotic promoter: how bacteria learn to respond to novel environmental signals. *Mol. Microbiol.* **19**, 1177-1184.
17. Fessard, V., T. Godard, S. Huet, A. Mourot and J. M. Poul. 1999. Mutagenicity of malachite green and leucomalachite green in *in vitro* tests. *J. Appl. Toxicol.* **19**, 421-430.
18. Díaz, E. and M. A. Prieto. 2000. Bacterial promoters triggering biodegradation of aromatic pollutants. *Cur. Opin. Biotechnol.* **11**, 467-475.
19. Gregory, P. 1993. Dyes and dyes intermediates, p. 544-545, *In* J. L. Kroschwitz (ed.), *Encyclopedia of Chemical Technology*, Vol. **8**, John Wiley & Sons, New York.
20. Harrelson, W. G. and R. P. Mason. 1982. Microsomal reduction of gentian violet. Evidence for cytochrome P-450 catalyzed free radical formation. *Mol. Pharmacol.* **22**, 239-242.
21. Henderson, A. L., T. C. Schmitt, T. M. Heinze and C. E. Cerniglia. 1997. Reduction of malachite green to leucomalachite green by intestinal bacteria. *Appl. Environ. Microbiol.* **63**, 4099-4101.
22. Indig, G. L., G. S. Anderson, M. G. Nichols, J. A. Bartlett, W. S. Mellon and F. Sieber. 2000. Effect of molecular structure on the performance of triarylemthane dyes as therapeutic agents for photochemical purging of autologous bone marrow grafts from residual tumor cells. *J. Pharm. Sci.* **89**, 88-99.
23. Jone, J. J. and J. O. Falkinham. 2003. Decolorization of malachite green and crystal violet by waterborne pathogenic mycobacteria. *Antimicrob. Agents Chemother.* **47**, 2323-2326.

24. Kean, B. H. and D. W. Haskins. 1978. Drugs for intestinal parasitism, p. 371-381, *In* W. Modell (ed.), *Drugs of choice*, Mosby Co., St. Louis.
25. Kingsland, G. V. and J. Anderson. 1976. A study of the feasibility of the use of gentian violet as a fungistat for poultry feed. *Poult. Sci.* **55**, 852-857.
26. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3**, 208-218.
27. Nelson, C. R. and R. A. Hites. 1980. Aromatic amines in and near the Buffalo River. *Environ. Sci. Technol.* **14**, 1147-1149.
28. Rao, K. V. K. 1995. Inhibition of DNA synthesis in primary rat hepatocyte cultures by malachite green: a new liver tumor promoter. *Toxicol. Lett.* **81**, 107-113.
29. Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. Sasakawa, C. and M. Yoshikawa. 1987. A series of Tn5 variants with various drug-resistance markers and suicide vector for transposon mutagenesis. *Gene* **56**, 283-288.
31. Schell, M. A. 1993. Molecular biology of the LysR family of transcriptional regulators. *Annu. Rev. Microbiol.* **47**, 597-626.
32. Shin, K.-S. and C.-J. Kim. 1998. Decolorization of artificial dyes by peroxidase from the white-rot fungus *Pleurotus ostreatus*. *Biotechnol. Lett.* **20**, 569-572.
33. Vasdev, K., R. C. Kuhad and R. K. Saxena. 1995. Decolorization of triphenylmethane dyes by the birds nest fungus *Cyathus bulleri*. *Curr. Microbiol.* **30**, 269-272.

초록 : *Citrobacter* sp.에서 crystal violet와 malachite green 색소분해에 관여하는 유전자들의 동정

이영미 · 장문선 · 김석조 · 최용락 · 조영수 · 이영춘*
(동아대학교 응용생명공학부)

Crystal violet와 malachite green색소 분해에 관여하는 유전자들을 규명하기 위하여 색소분해능을 가진 *Citrobacter* sp.의 염색체 DNA속에의 transposon 도입에 의해 생성된 무작위 변이주들이 분리되었다. 이들 변이주들로부터 두가지 색소분해능을 소실한 14개의 변이주들이 선별되었고, 이들로부터 염색체 DNA를 분리하여 *Eco*RI으로 절단한 후 Tn5 단편을 probe로하여 Southern hybridization을 행한 결과, 염색체 DNA상의 각각 다른 부위에 Transposon이 Single 삽입된 5개의 변이주 (Cmg2, Cmg6, Cmg8, Cmg11, Cmg12)가 최종적으로 분리되었다. 이들 변이주들의 Transposon 삽입부위 주위의 염기서열과 이로부터 유추되는 아미노산서열을 database상에 등록되어 있는 유전자의 염기서열과 단백질의 아미노산 서열에 대한 상동성을 비교한 결과, Cmg2는 대장균 maltose trnasporter (Mal G)이고, Cmg6은 LysR-type 전사조절 단백질이며, Cmg12는 산화환원효소를 코드하는 유전자인 것으로 알려졌고, 나머지 Cmg8과 Cmg11은 아직까지 기능이 알려져 있지 않은 단백질을 코드하는 유전자인 것으로 임이 판명되었다.