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NOTE

Isolation of *Citrobacter* sp. Mutants Defective in Decolorization of Brilliant Green by Transposon Mutagenesis

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To identify genes involved in the decolorization of brilliant green, we isolated random mutants generated by transposon insertion in brilliant green-decolorizing bacterium, *Citrobacter* sp. The resulting mutant bank yielded 19 mutants with a complete defect in terms of the brilliant green color removing ability. Southern hybridization with a Tn*5* fragment as a probe showed a single hybridized band in 7 mutants and these mutants appeared to have insertions at different sites of the chromosome. Tn*5*-inserted genes were isolated and the DNA sequence flanking Tn*5* was determined. By comparing these with a sequence database, putative protein products encoded by *bg* genes were identified as follows: *bg 3* as a LysR-type regulatory protein; *bg 11* as a MalG protein in the maltose transport system; *bg 14* as an oxidoreductase; and *bg 17* as an ABC transporter. The sequences deduced from the three *bg* genes, *bg 2*, *bg 7* and *bg 16*, showed no significant similarity to any protein with a known function, suggesting that these three *bg* genes may encode unidentified proteins responsible for the decolorization of brilliant green.

Key words: Brilliant green, *Citrobactor sp.*, Decolorization; Transposon mutagenesis

Triphenylmethane dyes are aromatic xenobiotic compounds, and are used extensively in the textile industry to dye nylon, wool, silk and cotton, and in paper and leather industries (Gregory, 1993). Some of these dyes are used as biological stains and in veterinary medicine (Kingsland and Anderson, 1976; Kean and Haskin, 1978). Significantly, some of these dyes have been shown to be mutagens, mitotic poisons and clastogens (Au *et al.*, 1978; 1979). The degradation of dyes has received considerable attention from the viewpoint of treating industrial wastewater containing such dyes. To eliminate the colored effluents in wastewater, several physico-chemical methods, including adsorption, flocculation, photolysis, ionization, ozonation, oxidation and reduction have been used (Reife, 1993; Cooper, 1995). Although these methods are effective, they are generally expensive, of limited applicability and produce large amount of sludge. Therefore, interest has focused on the microbial biodegradation of dyes, as a potential alternative.

Several triphenylmethane dye-degrading microorganisms have been reported and their characteristics reviewed (Banat *et al.*, 1996; Azmi *et al.*, 1998). The mechanism of the biodecolorization of triphenylmethane dyes has been elucidated in fungi (Bumpus and Brock, 1988; Vasdev *et al.*, 1995; Azmi *et al.*, 1998; Shin and Kim, 1998), but not in bacteria. Triphenylmethane dyes were degraded by a ligninolytic culture of *Phanerochaete chrysosporium*, and its initial oxidation was found to proceed via *N*-demethylation catalyzed by lignin peroxidase (Bumpus and Brock, 1988). Also, the decolorization of triphenylmethane dyes was found to be carried out by laccase in extracellular fluid from *Cyathus bulleri* (Vasdev *et al.*, 1995), and by a peroxidase from *Pleurotus ostreatus* (Shin and Kim, 1998). Moreover, the structural genes encoding lignin peroxidase and laccase have been cloned and characterized (Gold and Alic, 1993; Mayer and Staples, 2002). Although several triphenylmethane dye-decolorizing bacteria have been isolated (Azmi *et al.*, 1998), no information is available on the genes involved in the decolorization of triphenylmethane dyes.

Recently, we isolated a new potent bacterium, *Citrobacter* sp., which has a higher decolorizing ability, even at high triphenylmethane and azo dye concentration than any other microorganism reported to date (An *et al.* 2002).

To understand the molecular mechanism of triphenylmethane dye-decolorization by bacteria, we used transpo-

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son mutagenesis with Tn*5* to investigate the genes responsible for the decolorization of triphenylmethane dyes in *Citrobacter* sp. In this study, we isolated and characterized the genes of seven mutants defective in the decolorization of brilliant green, an *N*-methylated triphenylmethane dye, which is used extensively in the textile industry (Gregory, 1993), and which has been found to cause dermatitis (Hatch and Maibach, 1995). This is the first report on a bacterial molecular genetic approach to triphenylmethane dye-decolorization.

E. coli MC1061[pR388(ts)::Tn*5*] (Sasakawa and Yoshikawa, 1987) used as a Tn*5* donor in transposon mutagenesis was grown at 30°C in Luria-Bertani (LB) medium containing trimethoprim (Tp, 12.5 μg/ml) and kanamycin (50 μg/ml). Chemicals including antibiotics and restriction enzymes were purchased from Sigma (USA) and Takara Korea (Korea). *Citrobacter* sp. was grown at 37°C in LB medium containing streptomycin (50 μg/ml). *E. coli* MC1061 [pR388 (ts)::Tn*5*] and *Citrobacter* sp. were grown to an OD_{660nm} of 0.6. 500 ml of each strain was taken out, mixed, and 30 ml of the mixture was spotted onto LB agar plates. After incubation at 30°C for 12 h, the cells were collected by scraping, and transconjugants were selected after incubating at 30°C for 24 h on M9 agar plates containing trimethoprim (12.5 μg/ml) and streptomycin (50 μg/ml). From transconjugants, strains lacking pR388 (ts)::Tn*5* were isolated by incubating at 42°C for 15 h in LB agar plate containing kanamycin (50 μg/ml). Colonies were picked and transferred onto LB agar plates containing kanamycin (50 μg/ml) and brilliant green (300 μM). After incubation at 37°C for 3 days, colonies lacking decolorizing activity were selected and confirmed by shaking incubation in LB medium containing kanamycin (50 μg/ml) and brilliant green (300 μM). By screening about 35,000 colonies, we isolated 19 mutants which did not decolorize brilliant green. These mutants were presumed to harbor affected dye-decolorizing genes, and were called *bg* mutants. Southern hybridization (Sambrook and Russell, 2001) using Tn*5* DNA fragment as a probe showed a single hybridized band for 7 mutants, indicating that these mutants contained single transposon insertions (Fig. 1). The remaining harbored two or three transposon insertions in the genome. Therefore, 7 mutants harboring only single transposon insertion were characterized further.

To determine the functions of the *bg* genes, *Xho*I, *Hin*dIII and *Pst*I fragments containing Tn*5* DNA fragment from mutants harboring only a single transposon insertion were subcloned into the corresponding sites of pBluescript SK(+) or pUC 118 and 119. The DNA sequence flanking Tn*5* was then determined by using a synthetic primer (5'-CTGTCTCTTGATCAGATCT-3') complementary to the distal end of Tn*5*. The obtained sequence was translated, and amino acid sequences were inferred from each open reading frame that had been interrupted by Tn*5*, and compared with protein sequences in the protein database using

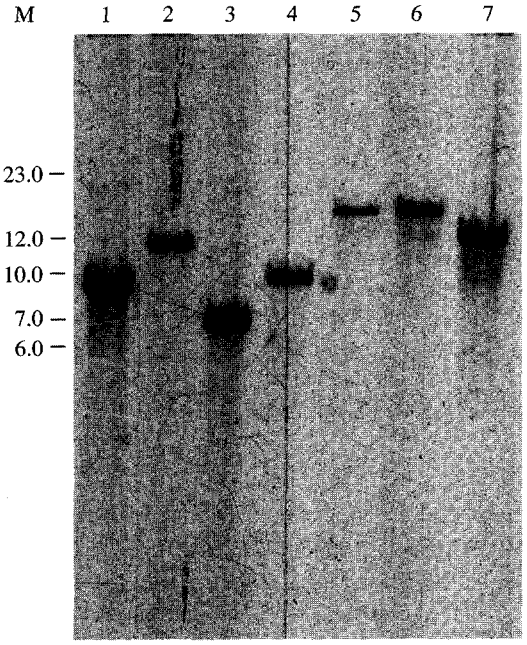

Fig. 1. Southern blot analysis using Tn*5* DNA as a probe. Chromosomal DNA was isolated from each strain, digested with *Eco*RI, and electrophoresed in a 0.5% (w/v) agarose gel. DNA was blotted on a nylon membrane and hybridized with ^{32}P-labeled Tn*5* DNA as a probe. Lanes 1 to 7, chromosomal DNA isolated from the mutant strain: *bg 2* (lane 1), *bg 3* (lane 2), *bg 7* (lane 3), *bg 11* (lane 4), *bg 14* (lane 5), *bg 16* (lane 6) and *bg 17* (lane 7). M, DNA marker digested with *Hin*dIII.

Table 1. Characterization of brilliant green decolorizing-defective mutants containing a single transposon insertions

Mutants	Homologous protein[a]	Organism	Putative function or location	% identity (no. of amino acids)[b]
bg 2	NP_415105	*E. coli* K-12	Unknown	78 (36)
bg 3	NP_418042	*E. coli* K-12	Transcriptional regulator (LysR)	84 (37)
bg 7	NP_706420	*Shigella flexneri* 2a strain 2457T	Unknown	88 (76)
bg 11	MalG	*E. coli* O157:H7 EDL933	Transport system (permease)	53 (119)
bg 14	AAC74663	*E. coli* K-12	Oxidoreductase	63 (69)
bg 16	NP_415882	*E. coli* K-12	Unknown	91 (47)
bg 17	C48399	*E. coli* K-12	ABC transporter	75 (77)

[a]Using BLAST program of the National Center for Biotechnology Information (NCBI), homologues with the highest sequence identity is shown. In the absence of a clear protein designation, homologues are listed by accession numbers.
[b]Number of amino acids used in the comparison of amino acid identity is indicated in parentheses.

BLAST program. The putative function of each of the *bg* genes was deduced by similarity with known proteins. The results are shown in Table 1.

Sequence analysis of the flanking regions of the transposon in *bg 3* showed high homology (84% identity) with a putative LysR-type transcriptional regulator of *E. coli* K-12 (Blattner *et al.*, 1997). The success of the aromatic catabolic pathway depends on two major elements: the catabolic enzymes leading to mineralization of the compound; and regulatory elements (de Lorenzo and Pérez-Martín, 1996). Regulatory proteins and regulated promoters are the key elements that control the transcription of catabolic operons. LysR-type regulatory protein is a transcriptional regulator that controls the expressions of aromatic catabolic pathways (Schell, 1993; Díaz and Prieto, 2000). Thus, *bg 3* may encode a regulatory protein that controls the transcription of the genes associated with brilliant green catabolism in *Citrobacter* sp.

The amino acid sequence deduced from *bg 11* showed 53% identity with MalG protein in the maltose transport system of *E. coli* O157:H7 (Perna *et al.*, 2001). In *E. coli*, MalG and MalF are hydrophobic inner membrane components, which mediate the energy-dependent translocation of substrate into the cytoplasm (Dassa and Muir, 1993). The binding protein-dependent maltose transport system of enterobacteria (MalFGK2), a member of the ABC transport superfamily, is composed of two integral membrane proteins, MalF and MalG, and two copies of an ATPase subunit (MalK) which hydrolyze ATP and thus energize the translocation process (Boos and Shuman, 1998). The amino acid sequence deduced from the DNA sequences of *bg 17* had significant identity (75%) with an ATP-binding cassette (ABC) transport protein of *E. coli* K-12 (Blattner *et al.*, 1997). ABC transporter is a major system of bacteria that participate in the export of a wide variety of substances, such as proteins, polysaccharides, antibiotic, and growth inhibitors (Faith and Kolter, 1993). From these observations, it is likely that *bg 11* and *bg 17* encode proteins that act as transporters associated with the decolorization of brilliant green.

The amino acid sequence deduced from *bg 14* showed high sequence homology (63% identity) with the putative oxidoreductase of *E. coli* K-12 (Blattner *et al.*, 1997). Malachite green, a triphenylmethane dye, is decolorized by enzymatic reduction in intestinal microflora (Henderson *et al.*, 1997). Decolorization and biodegradation of triphenylmethane dyes including brilliant green by a fungus *P. chrysosporium* occur by oxidative reaction via *N*-demethylation catalyzed by lignin peroxidase (Bumpus and Brock, 1988). *P. chrysosporium* could also degrade these dyes under nonligninolytic conditions, suggesting that another mechanism for degrading these dyes exists in this fungus (Bumpus and Brock, 1988). These results suggest that *bg 14* is the gene encoding a protein responsible for the oxidation or reduction of brilliant green in *Citrobacter* sp. On the other hand, the sequence analyses of *bg 2*, *bg 7* and *bg 16* showed no significant similarity to any protein with a known function, but they did show more than 78% identity with hypothetical proteins of other bacteria including *E. coli*.

Based on these results, the cloning, sequencing and expression of the full-length DNA of Tn*5*-inserted genes should help to clarify the exact functions of the genes responsible for the decolorization of brilliant green. The cloning of the open reading frames of these genes using fragments of *bg* genes obtained in this study is currently being progressed.

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