Korean Journal of Life Science
Vol. 10. No. 4. 333~339, 2000

Isolation and Characterization of *Citrobacter* sp. Mutants Defective in Decolorization of Crystal Violet

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Abstract

To identify genes involved in the decolorization of crystal violet, we isolated random mutants generated by transposon insertion in crystal violet-decolorizing bacterium, *Citrobacter* sp. The resulting mutant bank yielded mutants with six distinct phenotypes, and Southern hybridization with a Tn5 fragment as a probe showed a single hybridized band in the mutants Ctg 2, 5 and 6, whereas two and three bands were detected in Ctg1, 4 and 3, respectively. Tn5-inserted genes were isolated and the DNA sequence flanking Tn5 was determined. From comparison with a sequence database, putative protein product encoded by *ctg* 5 was identified as *E. coli* maltose transporter (Mal G) homolog, whereas the deduced amino acid sequence of the other *ctg* genes did not show any significant similarity with any DNA or protein sequence. Therefore, these results indicate that the other *ctg* genes except *ctg* 5 encode new proteins responsible for decolorization of crystal violet.

Key words – Transposon mutagenesis, Decolorization, Crystal violet, *Citrobactor* sp.

Introduction

Triphenylmethane dyes are used extensively in textile industries for dying nylon, wool, silk, cotton [16], 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.

Crystal violet (CV) is a triphenylmethane dye that has been used extensively in human and veterinary medicine as biological stain [19,20], 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 [25]. Recently, CV exhibited pronounced phototoxicity toward L1210 leukemia cells but comparatively small toxic effects toward normal hematopoietic cells [18].

Several triphenylmethane dye-degrading microorganisms have been reported and their characteristics have been reviewed recently [5,7]. Most of these microorganisms decolorized crystal violet. The mechanism for biodecolorization of crystal violet has been elucidated by fungi [5,10,28,31], but not by bacteria. CV was degraded by lignolytic 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* [31], and by peroxidase from *Pleurotus ostreatus* [28].

Very recently, we isolated a new potent bacterium,

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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 crystal violet in *Citrobacter* sp. In this study, we isolated six decolorizing-defective mutants and characterized their genes. This is the first report on molecular genetic approach of dye-decolorization by bacteria.

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 [27]. *E. coli* MC1061 [pR388(ts)::Tn5] was grown at 30℃ in LB medium containing trimethoprim (Tp, 12.5 ㎍/ml) and kanamycin (50 ㎍/ml). *Citrobacter* sp. was grown at 37℃ in LB medium containing streptomycin (50 ㎍/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 ㎍/ml) and streptomycin (50 ㎍/ml). From transconjugants strains lacking pR388(ts)::Tn5 were isolated by incubation at 42℃ for 15 h in LB agar plate containing kanamycin (50 ㎍/ml). Colonies were picked and transferred onto LB agar plate containing kanamycin (50 ㎍/ml) and crystal violet (245 ㎛M). After incubation at 37℃ for 3 days, colonies lacking decolorization activity were selected and confirmed by shaking incubation in LB medium containing kanamycin (50 ㎍/ml) and crystal violet (245 ㎛M). Also, it was confirmed, by Southern hybridization [26] 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 [26]. Chromosomal DNAs of Citrobacter sp. and mutant strains were prepared by the method of Marmur [22]. 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.* [26]. 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 *ctg 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 *ctg* gene was deduced from the similarity of its product to known proteins.

Results and Discussion

Isolation of crystal violet-nondecolorizing mutants

To investigate the molecular mechanism of dye decolorization, we tried to isloate crystal violet-nondecolorizing mutants generated by transposon insertion. Transposon mutagenesis was carried out by conjugative transfer of Tn5 from *E. coli* MC1061 [pR388(ts)::Tn5] to *Citrobacter* sp., and crystal violet-nondecolorizing mutants were screened by replica plating on the plates containing crystal violet. From a screening of about 12,000 colonies, we isolated six mutants which did not decolorize crystal violet. These mutants should be affected in dye-decolorizing genes, and they were called *ctg* mutants.

Characterization of ctg mutants

The presence of Tn5 insert in each of the mutant strains was confirmed by Southern blot analysis (Fig. 1). The Tn5 probe hybridized with one band in the mutants Ctgs 2, 5 and 6 when DNA was digested with *Eco*RI, which did not cut Tn5, whereas two and three bands were detected in Ctgs1, 4 and Ctg3, respectively. These results show that mutants Ctgs 2, 5 and 6 have single transposon insertion, whereas mutants Ctgs1, 4 and Ctg3 have two and three transposon insertions, respectively. Also, Fig. 1 revealed that restriction digest of mutants Ctgs 1, 3, 4 and 5 yielded partially Tn5 hybridized bands of similar sizes: about 20 kb fragment in Ctg 1 to that in Ctg 3, 8 kb in Ctg 1 to that in Ctg 4, and 18 kb in Ctg 4 to that in Ctg 5. This similarity in band size indicates a similarity between the structure of the genes in which the Tn5 is inserted in each mutants.

Identification of *ctg* genes

To elucidate possible functions of *ctg* genes, we cloned the Tn5-inserted genes except mutant Ctg 3, and the DNA sequence flanking the transposon was determined. The possible function of each *ctg* gene was inferred from a comparison of the translated amino acid sequences in a database.

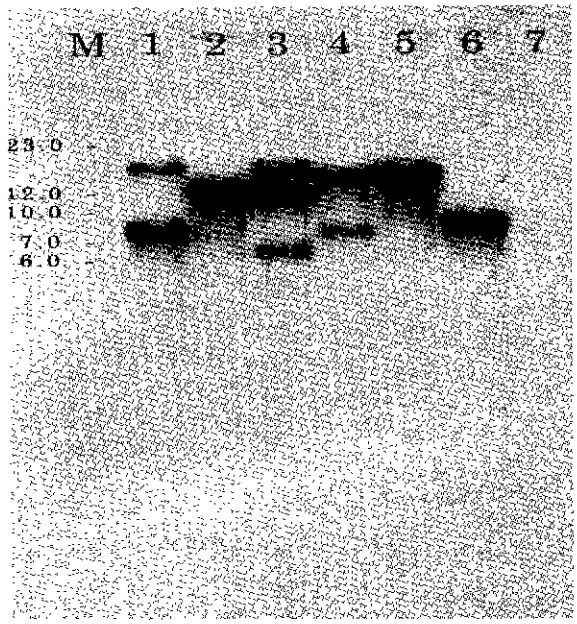

Fig. 1. Southern blot analysis with Tn5 DNA as a probe. Chromosomal DNA was isolated from each strain, digested with *Eco*RI, and electrophoresed on a 0.5 % (w/v) agarose gel. DNA was blotted on a nylon membrane and hybridized with ^{32}P-labeled Tn5 DNA as a probe. Lanes 1 to 6, chromosomal DNA isolated from the mutant strain: Ctg 1 (lane 1), Ctg 2 (lane 2), Ctg 3 (lane 3), Ctg 4 (lane 4), Ctg 5 (lane 5) and Ctg 6 (lane 6). Lane 7, chromosomal DNA isolated from wild type strain. M, DNA marker digested with *Hind*III and 1 kb ladder.

The amino acid sequence deduced from the DNA sequence of *cgt* 5 had significant similarity to the sequence of COOH-terminal of MalG protein in maltose transport system of *E. coli* (Fig. 2) [13]. In *E. coli*, MalG and MalF are hydrophobic inner membrane components mediating the energy-dependent translocation of substrate into the cytoplasm [13]. The binding protein-dependent maltose transport system of enterobacteria (MalFGK2), a member of the ATP-binding cassette (ABC) transport superfamily, is composed of two intergal membrane proteins, MalF and MalG, and of two copies of an ATPase subunit, MalK,

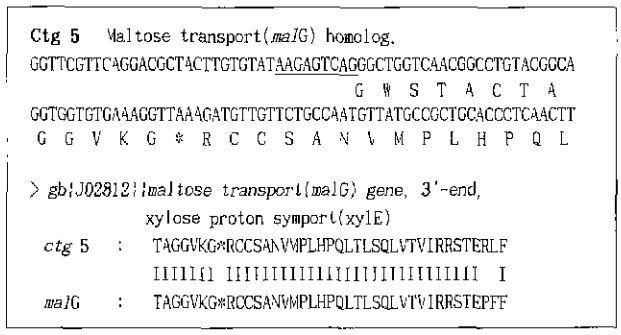

Fig. 2. Sequence ananlysis of *ctg* 5 gene. The nucleotide sequence of *ctg* 5 gene flanking Tn5 was determined, and the deduced amino acid sequence was compared with database sequences. Tn5 insertion sites are underlined. The symbol I indicates the identical amino acid.

which hydrolyze ATP, thus energizing the translocation process [8]. In addition, an extracellular (periplasmic) substrate-binding protein (MalE) is required for activity [23]. In the maltose system, MAlF and MalG, both reported to interact with either one of the two domains of MalE [17]. Ligand translocation and ATP hydrolysis are dependent on a signaling mechanism originating from the binding protein and traveling through MalF/MalG [14]. Mourez *et al.* reported that mutations in a conserved motif (EAA loop) of the membrane-spanning subunits MalF and MalG affected slightly or did not affect transport, and determined a completely defective phenotype when presented together, suggseting that EAA regions of MalF and MalG may interact during transport, and constitute a recognition site for the ABC ATPase helical domain [24].

Considering the above informations, at present, although it is not clear whether the ctg5 gene encodes a protein acting as a transport of CV, this gene may encode a transporter protein containing an ATP-binding cassette (ABC transporter).

DNA sequence analysis showed that 8 kb of *ctg* 1 is identical to that of *ctg* 4, and their deduced amino acid sequences did not show any significant similarity with any DNA or protein sequence in database (Fig. 3).

As shown in Fig. 4, the deduced amono acids of *ctg*2 had a complete sequence homology with 10. 8 kD protein in Tpia-FPR intergenic region of *E. coli*. Because the function of this protein in *E. coli* is not known, we do not know the function of *ctg*2 gene.

Sequence comparison of *ctg*6 showed that it is GTP-binding protein era operon homolog of *E. coli* (Fig. 5) [1]. The *era* (for *E. coli ras*) gene is a member of the *rnc* operon, which contains *rnc*, *era*, and *recO* [30]. The *rnc* gene encodes the protein RNase III, which is an RNA endonuclease that plays a role in the processing of several

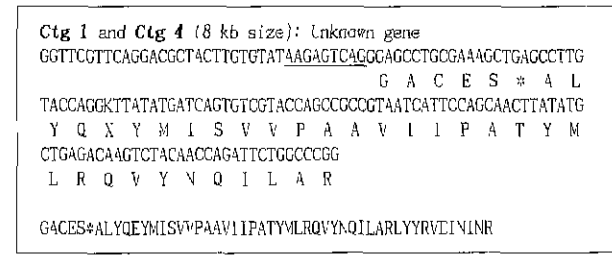

Fig. 3. Sequence ananlysis of *ctg* 1 and 4 genes. The nucleotide sequence of each *ctg* gene flanking Tn5 was determined, and the deduced amino acid sequence was compared with database sequences. Tn5 insertion sites are underlined.

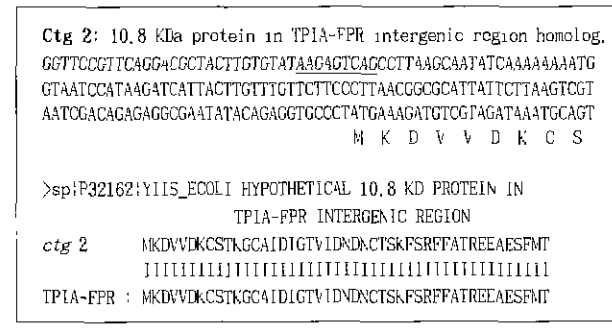

Fig. 4. Sequence ananlysis of *ctg* 2 gene. The nucleotide sequence of *ctg* 2 gene flanking Tn5 was determined, and the deduced amino acid sequence was compared with database sequences. Tn5 insertion sites are underlined. The symbol I indicates the identical amino acid.

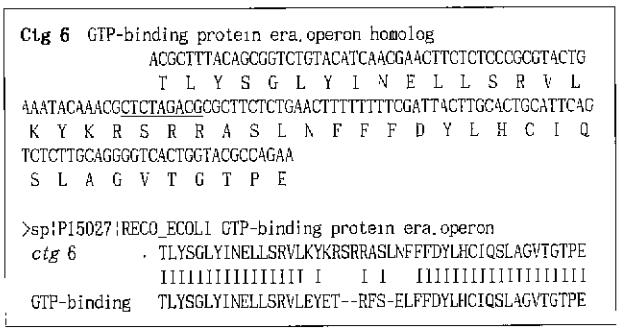

Fig. 5. Sequence ananlysis of *ctg* 6 gene. The nucleotide sequence of *ctg* 6 gene flanking Tn5 was determined, and the deduced amino acid sequence was compared with database sequences. Tn5 insertion sites are underlined. The symbol I indicates the identical amino acid.

RNA transcripts in *E. coli* [12]. The *era* gene encodes a GTPase of unknown function. The RecO protein is involved in the RecF recombination pathway [32]. Of the three genes, only the *era* gene is required for viability in *E. coli* [30]. Proteins that comprise the GTPase superfamily have been identified in organisms ranging from *Escherichia coli* to humans [9]. These proteins are turned on by the binding of GTP and off by the hydrolysis of GTP to GDP, there by acting as a molecular switch [9]. GTPase are involved in diverse cellular processes such as signal transduction, protein translocation, and cell cycle regulation [9]. Purified Era protein binds guanine nucleotides specifically and is able to hydrolyze GTP to GDP [11]. The protein also autophosphorylates in a GTP-dependent manner in vitro [29]. Although its function is unknown, this phosphorylated form of Era has been suggested to be the active form of the protein [29]. Era has been found to be associated with an inner membrane fraction in vitro [21].

Considering the above informations, at present, although it is not clear whether the *ctg*6 gene encodes a Era protein having unknown function, this gene may encode a GTP-binding protein associated with decolorization of crystal violet.

The cloning, sequencing and expression of Tn5-tagged genes should help to clarify the exact functions of their genes responsible for the decolorization of crystal violet. In our laboratory, cloning of open reading frame (ORF) of these genes using fragments of *cgt* genes obtained in this study are in progress.

Acknowledgements

The authors wish to acknowledge the financial support (Grant No. 1998-019-G00015) of the Korea Research Foundation made in program year of 1998.

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초록 : Crystal violet 색소분해능이 소실된 *Citrobacter* sp.의 분리 및 특성

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Crystal violet 색소 분해에 관여하는 유전자들을 규명하기 위하여 색소분해능을 가진 *Citrobacter* sp.의 염색체 DNA속에의 transposon 도입에 의해 생성된 무작위 변이주들이 분리되었다. 이들 변이주들로부터 색소분해능을 소실한 6개의 변이주(Ctg 1-6)들이 선별되었고, 이들로부터 염색체 DNA를 분리하여 *Eco*RI으로 절단한 후 Tn5 단편을 probe로하여 Southern hybridization을 행한 결과, 염색체 DNA상에 Transposon이 Single 삽입된 변이주로서는 Ctg2, Ctg5, Ctg6 변이주이고, 2군데 삽입된 변이주는 Ctg1과 Ctg4이며, 나머지 Ctg3는 3군데 Transposon이 삽입된 변이주로서 얻어졌다. 이들 변이주들의 Transposon 삽입부위 주위의 염기서열과 이로부터 유추되는 아미노산서열을 database상에 등록되어 있는 유전자의 염기서열과 단백질의 아미노산 서열에 대한 상동성을 비교한 결과, Ctg5는 대장균 maltose trnasporter (Mal G)의 유사체로서 판명되었고, Ctg1과 Ctg4의 8 kb 크기의 단편은 동일 유전자로서 지금까지 database에 등록되어있지 않은 새로운 유전자임이 판명되었다. 또한 Ctg5는 대장균 유래의 기능이 알려져 있지 않은 10.8 kD의 단백질을 코드하는 유전자임이 확인되었고, Ctg6는 GTP-binding protein era operon임이 확인되었다.